

FORM PTO-1390 (REV 10-95)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371		UNIMD 4		
		U.S. APPLICATION NO. (If known, see 37 CFR §1.5) 09/936565		
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED		
PCT/US00/06588	15 MARCH 2000	15 MARCH 1999		
TITLE OF INVENTION SURFACE LOCALIZED COLLIGIN/HsP47 IN CARCINOMA CELLS				
APPLICANT(S) FOR DO/EO/US SAUK, John, J.				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)). 				
Items 11. to 16. below concern document(s) or information included:				
<ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included. 13. <input type="checkbox"/> A FIRST preliminary amendment. <ol style="list-style-type: none"> <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input type="checkbox"/> Other items or information: 				

U.S. APPLICATION NO. (Unknown, see 37 CFR §1.5) 09/4936565		INTERNATIONAL APPLICATION NO. PCT/US00/06588	ATTORNEY'S DOCKET NUMBER UNIMD 4
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)): Search Report has been prepared by the EPO or JPO..... \$860.00 International preliminary examination fee paid to USPTO (37 CFR §1.482)..... \$690.00 No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2))..... \$710.00 Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO..... \$1000.00 International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT = \$860.00			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. §1.492(e)).			
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	32 - 20 =	12	x \$ 18.00 \$216.00
Independent claims	13 - 3 =	10	x \$ 80.00 \$800.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$ 270.00	
		TOTAL OF ABOVE CALCULATIONS = \$1,876.00	
Reduction of 1/2 for filing by small entity, if applicable. Applicant qualifies.		(\$938.00)	
		SUBTOTAL = \$938.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. §1.492(f)).			
		TOTAL NATIONAL FEE = \$938.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). \$40.00 per property.			
		TOTAL FEES ENCLOSED = \$938.00	
		Amount to be refunded:	
		charged:	
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$938.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>13-3402</u> in the amount of <u>\$</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>13-3402</u> . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO: Customer Number 23,599			
 23599 <small>PATENT TRADEMARK OFFICE</small>		 Harry B. Shubin <small>NAME</small>	
Filed: 14 SEPTEMBER 2001		32,004 <small>REGISTRATION NUMBER</small>	
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PTO/PCT Rec'd 14 SEP 2000

SURFACE LOCALIZED COLLIGIN/Hsp47 IN CARCINOMA CELLS

This application claims the benefit of U. S. Provisional Application 60/124,481, filed March 15, 1999, which is incorporated by reference herein in its entirety.

Description of the Invention

5 This invention relates, *e.g.*, to colligin/Hsp47 molecules which are expressed on the surface of carcinoma cells and to the use of such expressed molecules as targets for, *e.g.*, therapeutic agents or imaging agents. The invention also relates to peptides which bind specifically to external domains of such surface-localized Hsp47 molecules.

10 The heat shock protein colligin/CBP2/Hsp47 (sometimes referred to herein as Hsp47) can act as a molecular chaperone for collagen. In normal cells, Hsp47 becomes closely associated with nascent chains of procollagen early during its translation and accompanies the protein from the endoplasmic reticulum (ER) to the Golgi, whereupon collagen dissociates and is secreted, and the chaperone is recycled back to the ER. It is disclosed herein that, surprisingly, in carcinoma cells Hsp47 is not efficiently recycled to the ER but, rather, is localized on (*e.g.*, leaks onto) the surface of the cells.

15 Examples 1 and 6 show that Hsp47 is localized on the surface of a number of carcinoma cell lines, including human oral squamous cell carcinoma lines, a murine epidermal cell line, breast cancer carcinoma lines and prostate cancer cell lines, but is not expressed on the surface of control, non-carcinoma cells. Thus, cell-surface localized Hsp47 can serve as a marker and/or "homing target" for carcinoma cells. Example 2 shows that at least of portion of surface localized Hsp47 is available for binding to, *e.g.*, a procollagen propeptide to which it normally binds in the intracellular environment. By targeting carcinoma cells with an agent that interacts specifically with available portions of Hsp47 expressed on the surface of the cells, one can deliver therapeutic agents efficiently, allowing for reduced doses required to achieve therapeutic effects and reduced side effects; and one can sensitively detect or image carcinoma cells, or carcinomas or tumors containing them, above a background of non-carcinoma cells.

20 This invention relates to a method for modulating a cell which expresses Hsp47 on its surface, comprising administering to the cell an effective amount of an agent which binds to an (at least one) external domain of Hsp47. The invention also relates to a

method for treating a patient suffering from a carcinoma in which Hsp47 is expressed on the surface of at least some of the carcinoma cells, comprising administering to the patient an effective amount of an agent which binds specifically to an external domain of Hsp47. In a preferred embodiment, the agent comprises a targeting moiety which is specific for Hsp47, and a therapeutic moiety, *e.g.*, a drug or toxic substance. In one embodiment, the targeting moiety comprises an antibody, or a fragment or variant thereof, which is specific for an external domain of Hsp47. In another embodiment, the targeting moiety comprises a peptide which is specific for an external domain of Hsp47, *e.g.*, a peptide having the consensus motif XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or the consensus motif HyXXXHyHyXXHyXXX (SEQ ID NO: 2). In a most preferred embodiment, the peptide has the sequence of one of SEQ ID NOs: 3 - 25. In yet another embodiment, the targeting moiety comprises a bacteriophage on whose surface is a peptide such as those disclosed above.

The invention also relates to a diagnostic imaging method of detecting a cell which expresses Hsp47 on its surface (*e.g.*, a carcinoma cell, or a carcinoma or tumor comprising such a cell), comprising contacting the cell with an agent comprising a targeting moiety that binds specifically to an external domain of Hsp47, and a detectable label (detectable moiety) (*e.g.*, a label which is detectable by MRI, X-Ray, gamma scintigraphy, CT scanning, or the like). The targeting moiety can comprise, *e.g.*, an antibody, a peptide or a bacteriophage as described above.

The invention also relates to a method of screening for an agent which binds specifically to a carcinoma in which Hsp47 is expressed on the surface of at least some of the carcinoma cells, comprising identifying an agent which binds specifically to an external domain of Hsp47. In a preferred embodiment, the agent is useful for treating a carcinoma in a patient or for diagnosing a carcinoma in a patient.

The invention also relates to kits for detecting a carcinoma cell or for treating a carcinoma cell, comprising a targeting moiety which binds specifically to an external domain of Hsp47.

The invention also relates to peptides which bind specifically to an external domain of Hsp47. In a preferred embodiment, the peptide contains the consensus motif XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or the consensus motif

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HyXXXHyHyXXHyXXX (SEQ ID NO: 2). In a most preferred embodiment, the peptide is one of SEQ ID NOs: 3 - 25.

Any type of cell which expresses Hsp47 on its surface can be modulated, treated and/or detected by the methods of the invention. In a preferred embodiment, the cell is 5 a carcinoma cell. The term "carcinoma" as used herein means any of the various types of malignant neoplasms derived from epithelial tissue in any of several sites, *e.g.*, skin, basal cells, large intestine, lung, colon, breast, bladder, oral, head and neck, larynx, nasopharynx, adrenal cortex, apocrine gland, cloaca, embryonal cells, kidney, liver, pancreas, or prostate. The term "carcinoma cell" as used herein applies to a carcinoma 10 cell, *in vivo* or *in vitro*, whether it occurs as an individual cell or in the context of a tissue, carcinoma in any stage of development, a tumor, a metastasis (including a micrometastasis), or the like.

Sarcoma cells are also encompassed by the invention. The term "sarcoma" as 15 used herein means any connective tissue neoplasm, formed by the proliferation of mesodermal cells. Among the many types of sarcomas encompassed by the invention are fibrosarcoma, rhabdosarcoma, neurofibrosarcoma and osteosarcoma. The term "sarcoma cell" as used herein applies to a sarcoma cell, *in vivo* or *in vitro*, whether it occurs as an individual cell or in the context of a tissue, a sarcoma in any stage of development, a tumor, a metastasis (including a micrometastasis), or the like. Methods 20 to study sarcoma cells, particularly in relation to the expression of Hsp47, are disclosed, *e.g.*, in Morino *et al.* (1997). *In Vivo* 11, 261-4; Morino *et al.* (1997). *In Vivo* 11, 17-21; Shirakami *et al.* (1995). *In Vivo* 9, 513-8; Shirakami *et al.* (1995). *In Vivo* 9, 509-12; Morino *et al.* (1995). *In Vivo* 9, 503-8; and Morino *et al.* (1994). *In Vivo* 8, 285-8.

Methods and reagents such as those discussed herein in relation to carcinoma 25 cells are, of course, applicable to any type of cell having surface expression of Hsp47, and to any physiological or pathological condition associated with such expression.

Hsp47 has been cloned and/or sequenced from a number of organisms, including, 30 *e.g.*, chicken (Hiroyashi *et al.* (1991). *Mol. and Cell Biol.* 11, 4036-4044); mouse (Takechi *et al.* (1992). *Eur. J. of Biochem.* 206, 323-329); and human (*e.g.*, Nakamura. accession number #D83174 in the DDJB/EMBL/GenBank databases; Ikegawa *et al.* (1995). *Cytogenet. Cell Genet.* 71, 182-186); and Jain *et al.* (1994). *Arch. Biochem. & Biophys.* 314, 23-30).

The term "Hsp47," as used herein, encompasses, *e.g.*, wild type Hsp47 from any mammalian source, preferably human, or a variant thereof. By "variant" of Hsp47 is meant, *e.g.*, any insertion, deletion, mutant form or substitution, either conservative or non-conservative, in either an internal or external domain of the protein, wherein such changes do not substantially alter the binding of the external domain to a targeting moiety as defined herein. By "conservative substitutions" is meant by combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Variants can include, *e.g.*, homologs, analogs, muteins and mimetics (mimotopes). Many types of protein modifications, including post-translational modifications, are included. See, *e.g.*, 5 modifications disclosed in U.S. Pat. No. 5,935,835. Hsp47 molecules which are modified so that they possess properties not normally associated with wild type Hsp47 are included, provided that an external domain can bind specifically to a targeting moiety 10 of the invention.

An agent which binds to Hsp47 expressed (*e.g.*, localized, located) on the surface 15 of a cell can bind to one or more of any portion(s) or fragment(s) of the Hsp47 molecule, of any length, which are available for binding. Such an available sequence is referred to herein as an "external domain" of the Hsp47 protein.

An agent or moiety which "binds specifically" to ("is specific for"; binds 20 "preferentially" to) an external domain of Hsp47 interacts with it, or forms or undergoes a physical association with it, in an amount and for a sufficient time to allow detection of the cell, or to modulate it (*e.g.*, to elicit a therapeutic response). By "specifically" or "preferentially" is meant that the agent has a higher affinity, *e.g.*, a higher degree of 25 selectivity, for an external domain of Hsp47 than for other molecules located on the surface of a cell. Similarly, an agent or moiety which binds specifically to a particular type of cell, *e.g.* a carcinoma cell, has a higher affinity, *e.g.*, a higher degree of selectivity, for that type of cell than for a normal (*e.g.*, non-carcinoma) cell, such that the binding allows the carcinoma cell to be detected and distinguished from a background 30 of normal cells, and/or allows for contacting a therapeutic moiety with a carcinoma cell but not (to a significant degree) with neighboring non-carcinoma cells. The affinity or degree of specificity can be determined by any of a variety of routine procedures. *e.g.*, competitive binding studies. See, *e.g.*, Czerwinski *et al.* (1998) *Proc. Nat. Acad. Sci.* **95**, 11,520-11,525 and Moe *et al.* (1998). *Phar. Res.* **23**, 31-38. The portion of an agent

which binds specifically to an external domain of Hsp47 can be referred to as a "targeting moiety," a "site-directed macromolecule," a "ligand" or an "affinity ligand."

Any agent or moiety which binds specifically to an external domain of Hsp47 is encompassed by the invention.

5 One type of targeting moiety comprises an antibody which binds specifically to an external domain of Hsp47. The term "antibody" as used herein can mean a polyclonal or, preferably, a monoclonal antibody, or a fragment (of any size) of a polyclonal or monoclonal antibody, *e.g.*, Fv, Fab', Fab, F(ab')₂, Fab'Fc, or the like. Single chain antibodies are also encompassed by the invention. Any form of immunoglobulin is included, *e.g.*, immunoglobulins A,D,E,G or M. One may wish to employ an intact antibody (*e.g.*, an IgG molecule), a bi- or multi-valent antibody which has specificity for at least two antigens, or a univalent antibody fragment. Antibodies of the invention can be isolated from natural sources; from hybridomas lymphomas, or the like; or they can be produced by synthetic and/or recombinant means. They can be partially or completely humanized, using conventional art-recognized procedures, as described, *e.g.*, in Jones *et al.* (1986). *Nature* 321,522; Riechmann *et al.*(1988). *Nature* 332, 323 ; Verhoyen *et al.*(1988). *Science* 239,1534; Carter *et al.*(1992). *Proc. Natl. Acad. Sci. USA* 89, 4285; Sandhu (1992). *Crit. Rev. Biotech.* 12,437; and Singer *et al.* (1933). *J. Immunol.* 150,2844.

20 Methods to screen for, isolate, purify, manipulate, etc. antibodies which exhibit the requisite specificity and/or affinity are conventional and routine in the art and are disclosed in, *e.g.*, U.S. Pat. 4,196,265; Roitt I., (1994). *Essential Immunology*, Blackwell Scientific Publications, London; Coligan *et al.*, *Current Protocols in Immunology*; Barnes *et al.*, in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (Humana Press 1992); Harlow *et al.*, *Antibodies: A Laboratory Manual*, page 726 (Cold Spring Harbor Pub. 1988); and *Current Protocols in Immunology*; Edited by John E. Coligan *et al.*, John Wiley & Sons, Inc.

30 Another type of targeting moiety comprises a peptide which binds specifically to an external domain of Hsp47. Such a peptide can be of any size or amino acid composition effective to bind specifically to an external domain of Hsp47, so as to allow the formation of a detectable entity or the production of a therapeutic response. In a preferred embodiment, the peptide is not full length collagen, and is not naturally

occurring collagen or a fragment thereof. In a preferred embodiment, the peptide is less than about 90 amino acids, *e.g.*, about 80, 70, 60, 50, 40, 30, 20 or 10 amino acids, most preferably about 12-16 amino acids. The invention also encompasses larger polypeptides, which can be, *e.g.*, as long as a full-length procollagen, providing that the polypeptide can bind to an external domain of Hsp47, so as to allow the formation of a detectable entity or the production of a therapeutic response.

Methods to determine if a peptide (or any other agent of interest) binds to a surface-expressed Hsp47 molecule are conventional and routine in the art. See, *e.g.*, Takemoto *et al.* (1992). *Arch. Biochem. and Biophys.* **296**, 323-329; Altemeyer *et al.* (1996). *International J. of Cancer* **69**, 340-349; Ferrarini *et al.* (1992). *International J. of Cancer* **51**, 613-619; Ullrich *et al.* (1986). *Proc. Natl. Acad. Sci.* **83**, 3121-3125; Vanbureskirk *et al.* (1989). *J. Exp. Med.* **170**, 1799-1809; Freedman *et al.* (1992). *J. of Neuroimmunology* **41**, 231-238; Sauk *et al.* (1997). *Connect. Tissue Res.* **37**, 105-119); and U.S. Patent 5,932,478. Such methods include, *e.g.*, detection of an agent which is tagged, directly or indirectly, with a fluorescent label by immunofluorescence microscopy, including confocal microscopy, or by flow cytometry (FACscan); detection of a radioactively labeled agent by autoradiography; electron microscopy; immunostaining; subcellular fractionation; or the like. Examples 1 and 6 illustrate some typical methods.

A number of peptides or peptide sequences have been identified which interact with intracellular Hsp47, and/or with the isolated protein. These peptides include, *e.g.*, the region of procollagen defined by the anti-propeptide antibody SP1.D8 (Hu *et al.* (1995). *J. of Cellular Biochemistry* **59**, 350-367); the N-propeptide region of procollagen of the $\alpha 1(I)$ -chain between residues 23-151, in particular residues 23-108; Gly-Xaa-Yaa sequences of procollagen, where X and Y are, independently, any amino acid; the (Pro-Pro-Gly)_n model sequences of collagen; and portions of gelatin (Nagata *et al.* (1988). *Biochem. Biophys. Res. Comm.* **153**, 428-434). Such peptides, or variants, mimetics (mimotopes), muteins, analogs or fragments thereof, can be tested readily for their ability to interact specifically with an external domain of Hsp47; and those which bind avidly and/or selectively can be used as targeting moieties in the invention.

By "variants" is meant any insertion, deletion, mutant form or substitution, either conservative or non-conservative, where such changes do not substantially alter the

binding of the peptide to an external domain of Hsp47. Types of variants include those discussed herein with reference to Hsp47 molecules. Among the modified peptides which are encompassed by the invention are peptidomimetics, which can be generated and tested by routine, conventional means. See, e.g., al-Obeidi *et al.* (1998). *Mol Biotechnol.* 9, 205-23; Kieber-Emmons *et al.* (1997). *Curr. Opin. Biotechnol.* 8, 435-41; Bowditch *et al.* (1996) *Blood* 88, 4579-4584; and Partidos *et al.* (1997) *Immunology Letters* 57, 113-116.

Methods to measure such binding constants/determine avidity are conventional in the art. See, e.g., Blond-Elguindi *et al.* (1993) *Cell* 75, 717-728. In a preferred embodiment, the binding constant (avidity) of the peptide or peptide region with Hsp47 is 15-45 μ m; most preferably, the binding constant is 20-30 μ m.

A technique for identifying peptides that can bind specifically and/or avidly to surface-expressed Hsp47 (external domains) is bacteriophage display (phage display) combined with affinity panning. See, e.g., Smith *et al.* (1993). *Methods Enzymol.* 217, 228-57; Smith *et al.* (1995). *J. Biol. Chem.* 270, 18,323-328; Kitamura *et al.* (1992). *Cell* 69, 823-831; Burkhardt *et al.* (1991). *Proc. Natl. Acad. Sci.* 88, 7410-7414; Pasqualini *et al.* (1996). *Brazilian J. of Med. & Biol. Res.* 29, 1151-1158; Pasqualini *et al.* (1996). *Molecular Psychiatry* 1, 423; Ruoslahti (1996). *Ann. Rev. of Cell & Developmental Biology* 12, 697-715; Ruoslahti (1997). *Kidney International* 51, 1413-1417; Hutchcroft *et al.* (1992). *J. Biol. Chem.* 267, 8613-8619; and Borst *et al.* (1993). *Immunological Reviews* 132, 49-84. Among the types of phage which can be used are single stranded filamentous phage, e.g., fd, F2, F5, M13, and variants thereof. Briefly, a set of random peptides of a chosen length (e.g., 7-mers or 12-mers) are cloned into and displayed on the surface of bacteriophage, and phage are selected which bind preferentially to cells bearing surface-expressed Hsp47, following one or more cycles of selection.

A demonstration of such panning is shown in Example 5. Phage-displayed 12-mer peptides identified in this Example contain, in general, either the consensus motif, XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or the consensus motif HyXXXHyHyXXHyXXX (SEQ ID NO: 2), where X is independently any amino acid and Hy is independently any hydrophobic amino acid. In a preferred embodiment, the hydrophobic amino acid is a large one, e.g., W (Trp), L(Leu) or F(Phe). Among the peptides having the consensus motif SEQ ID NO: 1 are those having the sequence of

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SEQ ID NO:3 to SEQ ID NO: 13, as shown in Table 1. These peptides can be characterized as predominantly hydrophobic peptides.

TABLE 1
PREDOMINANTLY HYDROPHOBIC PEPTIDES

5	WHWQWTPWSIQP WHYPWFQNWAMA WHWNGWKYPVVD FHWPTLYNMYIP FHWSWYTPSRPS	(SEQ. ID NO. 3) (SEQ. ID NO. 4) (SEQ. ID NO. 5) (SEQ. ID NO. 6) (SEQ. ID NO. 7)
10	WHWSYPLWGPLE NWTLPTAQFAYL VLIPVKALRAVVW TPQPNMMLRISP ANFTFFKLMPVS	(SEQ. ID NO. 8) (SEQ. ID NO. 9) (SEQ. ID NO. 10) (SEQ. ID NO. 11) (SEQ. ID NO. 12)
15	KVPPALPSPWTS	(SEQ. ID NO. 13)

Among the peptides having the consensus motif SEQ ID NO: 2 are those having the sequence of SEQ ID NO:14 to SEQ ID NO: 25, as shown in Table 2. These peptides can be characterized as predominantly hydrophobic peptides.

TABLE 2
PREDOMINANTLY HYDROPHILIC PEPTIDES

20	GLYMHPPTHTMR EGRSTLTSLTII SGAANQPSATSG KHNEQTFHPKVP TVLHSLAHQTFI AQSMMDVYSRQPF NTPTAPWHPGES RYMNDHKSPTDS SNAQEDVHDLSS TPSPNKSTVSPG KFMQAQAGMTHN LDSRYSLQAAMY	(SEQ. ID NO. 14) (SEQ. ID NO. 15) (SEQ. ID NO. 16) (SEQ. ID NO. 17) (SEQ. ID NO. 18) (SEQ. ID NO. 19) (SEQ. ID NO. 20) (SEQ. ID NO. 21) (SEQ. ID NO. 22) (SEQ. ID NO. 23) (SEQ. ID NO. 24) (SEQ. ID NO. 25)
25		
30		

The predominantly hydrophobic peptides identified in Example 5 surprisingly do not exhibit specific sequence identity to regions of procollagen, which is known to bind to Hsp47 but, taking into account the hydropathic profile of the amino acids (see discussion in Example 5), nevertheless map primarily to regions within the N-propeptide

region of collagen (residues 59-71) or the C-propeptide region (residues 1344-1445). The predominantly hydrophilic peptides also do not exhibit specific sequence identity to regions of procollagen, but generally map to regions within the helical region of procollagen.

5 Among the phage-displayed 7-mer peptides identified in Example 5 are those having the sequence of SEQ ID NO:26 to SEQ ID NO: 62, as shown in Table 3.

TABLE 3
7-MER PEPTIDES

10	GITSLLS FHSGWPQ TTNYYTN EPAHRSY SNAATEY KLSMTIP	(SEQ. ID NO. 26) (SEQ. ID NO. 27) (SEQ. ID NO. 28) (SEQ. ID NO. 29) (SEQ. ID NO. 30) (SEQ. ID NO. 31)
15	LVNMPTP SPNPWYG SLSTTQK TDTPRRQ KLTNTVL	(SEQ. ID NO. 32) (SEQ. ID NO. 33) (SEQ. ID NO. 34) (SEQ. ID NO. 35) (SEQ. ID NO. 36)
20	NWVPRTN TATSLQW KLPNVNS NVPYVVH DRFSPMP	(SEQ. ID NO. 37) (SEQ. ID NO. 38) (SEQ. ID NO. 39) (SEQ. ID NO. 40) (SEQ. ID NO. 41)
25	HFQPRHH HSTSTPH YVASPWQ FRYDTFP HNYLNLT	(SEQ. ID NO. 42) (SEQ. ID NO. 43) (SEQ. ID NO. 44) (SEQ. ID NO. 45) (SEQ. ID NO. 46)
30	ISQGTTP EFLPVQL HNYLNLT HPSLNKP HSTSVTQ	(SEQ. ID NO. 47) (SEQ. ID NO. 48) (SEQ. ID NO. 49) (SEQ. ID NO. 50) (SEQ. ID NO. 51)
35	YVASWPO ITVQKNT VAGNPLQ FTIPSNL NVMIKGQ	(SEQ. ID NO. 52) (SEQ. ID NO. 53) (SEQ. ID NO. 54) (SEQ. ID NO. 55) (SEQ. ID NO. 56)
40	QKPPPYD NVPYGVH AFLPSKL HFQPRHH	(SEQ. ID NO. 57) (SEQ. ID NO. 58) (SEQ. ID NO. 59) (SEQ. ID NO. 60)

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NTSPLEL
DFNYNPL

(SEQ. ID NO. 61)
(SEQ. ID NO. 62)

Peptides according to the invention include the SEQ IDs disclosed herein, as well as peptides which are extended by as many as, *e.g.*, 20 additional amino acids (*e.g.*, about 5 1, 3, 6, 9, 12, 15 or 18) on either or both ends of the peptide, provided that the extended peptide exhibits the requisite specificity/avidity for an external domain of Hsp47.

Preferably, a peptide of the invention is “isolated,” *e.g.*, is in a form other than it occurs in nature, *e.g.*, in a buffer, in a dry form awaiting reconstitution, as part of a kit, etc. In some embodiments, the peptide is substantially purified. The term “substantially purified”, as used herein, refers to a molecule, such as a peptide, that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a peptide, can be at least about 60%, by dry weight, preferably about 70%, 80%, 90%, 95% or 99% the molecule of interest. One skilled in the art can purify peptides using standard 10 protein purification methods and the purity of the peptides can be determined using standard methods including, *e.g.*, polyacrylamide gel electrophoresis (*e.g.*, SDS-PAGE), column chromatography (*e.g.*, high performance liquid chromatography (HPLC), and amino-terminal amino acid sequence analysis.

Once peptides exhibiting a specificity for surface-expressed Hsp47 are identified, 20 free energy calculations or other properties can be determined for each, and this information can be used to design other agents or drugs, *e.g.*, organic compounds, which bind preferentially to surface expressed Hsp47. Such methods are conventional. See, *e.g.*, Rejyo *et al.* (1996) *Proc. Natl. Acad. Sci.* **93**, 8945-8950; Gabius *et al.* (1998) *Phar. Res.* **15**, 23-30; and Selz *et al.* (1998) *Biophysical Jol.* **75**, 2332-2343.

25 Phage display/panning techniques can not only serve to identify peptides which can be used in the invention, but can also provide bacteriophage bearing such peptides on their surface which can be used in the invention. That is, a targeting moiety of the invention can be the peptide-bearing bacteriophage, itself. Such bacteriophage exhibit the advantage that they are readily internalized into the ER of cells to which they adhere. 30 See, *e.g.*, Example 6.

In one aspect of the invention, a targeting moiety as above is used to deliver a therapeutic moiety (e.g., a drug or toxic substance) to a cell which expresses Hsp47 on its surface (e.g., a carcinoma cell, for example one which constitutes part of a tissue or a tumor). A therapeutic agent of the invention preferably comprises a targeting moiety and, associated with it, a therapeutic moiety. A therapeutic agent can modulate a cell either positively or negatively, providing that it has a net therapeutic effect on the environment in which the cell resides (e.g., a tissue, tumor, metastasis, patient, or the like). By "modulate" is meant that any physiological response of the cell, e.g., a metabolic activity, a response to an internal or external environmental factor, a synthetic or catabolic process, activation, repression, etc., is altered. The therapeutic agent can achieve inhibition or suppression of growth, killing, destruction, elimination, control, modification, etc. of the cell or tissue. Cytostatic, cytolytic, cytotoxic, and carcinostatic effects are included. A therapeutic agent can suppress a neoplastic phenotype, or it can interfere with normal function of, or otherwise incapacitate, a cell to which it is delivered. In one embodiment, the therapeutic agent can prevent the establishment, growth or metastasis of a carcinoma, e.g., can prevent the recurrence of a carcinoma. Representative examples of antitumor agents, such as, e.g., immune activators and tumor proliferation inhibitors, are disclosed, e.g., in U.S. Patent No. 5,662,896. "Treatment" of, or the elicitation of a "therapeutic response" in, a cell, tissue, tumor, metastasis, patient, or the like, by a therapeutic agent (e.g., comprising a drug or toxic agent) is defined herein as an action which can bring about a response such as those discussed above. By an "effective amount" of a therapeutic agent is meant an amount which is sufficient to bring about such a response.

Methods to assay whether an agent elicits a therapeutic effect are routine and conventional, and can be performed *in vitro* or *in vivo*. A typical method for performing pharmacokinetics in an animal model is shown in Example 7. A typical animal model for testing the effect of an agent on tumors is the human oral squamous cell carcinoma xenograft model, is shown in Example 7. Among the factors which can be assayed are survival rate of the animal, reduction in size of a treated tumor, and the presence or absence of metastases, such as lymph node or lung metastasis.

Any of a wide variety of therapeutic moieties is encompassed by the invention, including therapeutic compounds which are used currently, but are delivered to cells by

other methods. The therapeutic moieties can be isolated from natural sources, or can be produced by synthetic and/or recombinant means, all of which are well-known to one of ordinary skill in the art. Among the drugs or therapeutic moieties which can be used in the invention are chemotherapeutic and/or cytotoxic agents such as, e.g., steroids, 5 antimetabolites, anthracycline, vincaalkaloids, neocarzinostatin (NCS), adriamycin, dideoxycytidine, cisplatin, doxorubicin, pirarubicin, melphalan and daunomycin, or the like. Methods to attach such moieties to targeting moieties are routine and conventional. For example, Example 7 illustrates methods to attach doxorubicin to an antibody or peptide targeting agent.

10 In one embodiment, the therapeutic moiety comprises a toxin such as, e.g., ricin (e.g., the A and/or B chain thereof, or the deglycosylated form), poisonous lectins, diphtheria toxin, exotoxin from *Psuedomonas aeruginosa*, abrin, modeccin, botulina toxin, alpha-amanitan, pokeweed antiviral protein (PAP, including PAPI, PAPII and PAP-S), ribosome inhibiting proteins, especially the ribosome inhibiting proteins of barley, wheat, corn, rye, or gelonin, or ribosome-inactivating glycoprotein (GPIR). 15 Fragments, subunits, muteins, mimetics, variants and/or analogues of such toxins are, of course, known to those of skill in the art and are encompassed by the invention. It is contemplated that all such variants or mutants which retain their toxic properties will be of use in accordance with the present invention.

20 Methods of selecting toxins and binding (e.g., associating, attaching or conjugating) them with a targeting moiety (e.g., a peptide or antibody), are routine and conventional in the art. See, e.g., U.S. patents 5,840,522; 5,079,163; 4,520,011; 5,667,786; 5,686,072; 4,340,535; 6,020,145; 5,254,342; 4,911,912; 4,450,154; and 5,928,873. Methods of attaching a peptide or polypeptide toxin to, e.g., a peptide or 25 antibody targeting moiety, include, for example, covalent binding, affinity binding, intercalation, coordinate binding and complexation. Covalent binding can be achieved either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent agents are useful in coupling protein molecules to other proteins, peptides or amine functions, etc. For example, the literature 30 is replete with coupling agents such as carbodiimides, diisocyanates, glutaraldehyde, diazobenzenes, and hexamethylene diamines. In some embodiments, one may first wish to derivatize the targeting moiety and then attach the toxin component to the derivatized

product. Suitable cross-linking agents for use in this manner include, *e.g.*, SPDP (N-succinimidyl-3-(2-pyridylthio)propionate) and SMPT (4-succinimidyl-oxycarbonyl- α -methyl α (2-pyridylthio)toluene). In one embodiment, a toxin and a targeting moiety can be covalently bonded by forming a disulfide bond between naturally occurring free thiol groups (*e.g.*, in the A chain of a ricin) and/or a thiol or activated disulfide group which has been introduced into an analogue of a peptide chain (*e.g.*, an analogue of gelonin having a cysteine available for disulfide bonding).

In another embodiment, the therapeutic moiety can comprise any of a variety of art-recognized radioisotopes or radionuclides. Methods of radiotherapy (nuclear medicine), in which cytotoxic doses of radioactivity are delivered to cells, are conventional in the art and are described, *e.g.*, in EP 481,526; U.S. Pat. 5,962,424; 10 Roeske *et al* (1990). *Int. J. Radiation Oncology Biol. Phys.* **19**, 1539-48; and Leichner *et al* (1993). *Med. Phys.* **20** (2 Pt. 2), 569-77. Such radioactive compounds can affect the 15 targeted cell as well as adjacent tumor cells which, for one reason or another, do not display Hsp47 on their surface. Further disclosure of the types of radioactive agents which can be used, and how to attach them to targeting moieties, is discussed below in reference to imaging agents. Among the most preferred radiation sources are Tc-99 and In-111.

In another embodiment, the therapeutic moiety can comprise an antibody, and can 20 be used as a basis for conventional types of immunotherapy, *e.g.*, as discussed below in regard to antibodies that are not associated with an additional therapeutic moiety.

Of course, combinations of the various therapeutic moieties can be coupled to one targeting moiety, thereby accommodating variable cytotoxicity. In another embodiment, two or more different therapeutic agents are administered together.

25 Many variations of treatment are encompassed by the invention. For example, it may be desirable to treat a patient preliminarily with an amount of a toxin (or other therapeutic agent) effective to generate an immune response, thereby providing systemic protection from the toxin to the patient. Subsequently, one can administer the toxin to the patient in an amount effective to kill tumor cells. See, *e.g.*, U.S. patent 5,667,786.

30 In another embodiment, an anti-Hsp47 antibody which is not associated with an additional therapeutic moiety can be used to treat, *e.g.*, a carcinoma, in a method of immunotherapy. Any of the types of antibodies described herein as targeting moieties

can be used for immunotherapy. Methods of immunotherapy are conventional and are described, *e.g.*, in U.S. Patents 6,015,567, 5,478,556 and 6,017,540.

In one such embodiment, the antibody brings about an effect by virtue of its association with Hsp47 on the cell surface. Example 3 demonstrates that cell surface 5 localized Hsp47 is associated with the tetraspanin protein, CD9, and thus can be involved in the interaction of cells with the cellular matrix. Therefore, an antibody directed against Hsp47 can modulate the interaction of a cell, *e.g.*, a carcinoma cell, with the intracellular matrix. Furthermore, cell surface expression of Hsp47 can be correlated with cell invasiveness and phagokinesis (mobility) which, in turn, can be correlated with 10 metastatic potential of a carcinogenic cell. Example 4 demonstrates a negative correlation in several cell lines between cell surface expression of Hsp47 and invasiveness/phagokinesis. Without wishing to be held to any particular mechanism, the invention encompasses methods of treatment with an anti-Hsp47 antibody to modulate 15 (increase or decrease a physiological activity or property of) a cell (*e.g.*, carcinoma cell, a tumor, etc.), for example to modulate invasion, migration/motility of tumor (*e.g.*, carcinoma) cells, and/or metastasis of a tumor cell (*e.g.*, carcinoma cell).

In cases in which Hsp47 cell surface expression is negatively correlated with tumor invasiveness and/or metastasis, the detection of surface-expressed Hsp47 can serve as the basis of a diagnostic method to identify tumors (*e.g.*, carcinomas) which are substantially non-metastatic and/or which are associated with a better prognosis than tumors on whose surface the expression of Hsp47 is substantially lower. 20

In another such embodiment, the antibody can elicit an immunological effect. Without wishing to be bound by any particular mechanism, the invention encompasses 25 situations in which an antibody recruits NK cells for antibody-mediated cell-mediated cytotoxicity. Bifunctional antibodies can bring effectors such as NK and T_c close to the tumor target. Many variations of immunotherapy will be evident to those of skill in the art. For example, two bispecific heteroantibodyconjugates, *e.g.*, anti-tumor/anti-CD3 and anti-tumor/anti-CD28, can be co-administered. Without wishing to be bound by any particular mechanism, it is proposed that two such heteroconjugates can act 30 synergistically to induce contact between a T-cell and a tumor to activate direct cytotoxicity even if the T-cell, itself, does not have conventional specificity for the tumor target.

5 Immunotherapy can be carried out by introducing an antibody directly into a patient, using conventional delivery methods and doses, such as those described herein for other therapeutic agents. Alternatively, one or more external domains of Hsp47, or fragments thereof, which contain an immune epitope can be introduced into a patient as a vaccine, in order to elicit a cell-mediated immune response. In a preferred embodiment, the external domain is "isolated," *e.g.*, is essentially free of transmembrane and cytoplasmic domains. The external domain or fragment thereof can be of any size, providing it comprises at least one immunoepitope. If desired, the external domain can be co-administered with, or conjugated to, one or more other reagents which can enhance the immune response, *e.g.*, a costimulatory molecule such as B7 or cytokines IFN γ , GMCSF, L2/4 and 7; an antigenic molecule, such as a peptide; or a suitable adjuvant.

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15 In another embodiment, a bacteriophage whose surface comprises a peptide which is specific for Hsp47 can serve as a vector for the implementation of gene therapy. Typical methods of phage display and affinity panning designed to obtain phage which can be used in such a method are described herein. A gene whose expression in a mammalian host is desired can be inserted into a mammalian expression cassette and then cloned into the phage genome. Cassettes for mammalian gene expression are conventional in the art, as are methods for cloning such cassettes into a nucleic acid of interest, *e.g.*, the DNA of a filamentous phage. Methods to clone and express genes are routine for one of ordinary skill in the art. See, *e.g.*, Sambrook, J. *et al* (1989). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel, F.M. *et al* (1995). *Current Protocols in Molecular Biology*, N.Y., John Wiley & Sons; and Davis *et al*. (1986), *Basic Methods in Molecular Biology*, Elsevier Sciences Publishing, Inc., New York. In particular, methods of using phage as gene delivery vehicles for mammalian cells are conventional and are described, *e.g.*, in Larocca *et al* (1999). *FASEB Journal* 13, 727-734; Barry *et al* (1996). *Nat. Biotechnology* 1282, 7711; Larocca *et al* (1998). *Hum. Gene Ther.* 9, 2393-2399; Poul *et al* (1999). *J. Mol. Biol.* 288, 203-11; and Kassner *et al* (1999). *Biochem. Biophys. Res. Comm.* 264, 921-8.

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30 For many of the molecular biology techniques referred to in this application, including isolating, cloning, modifying, labeling, manipulating, sequencing, and otherwise treating or analyzing nucleic acid and/or protein, see, *e.g.*, Hames *et al*. (1985),

Nucleic Acid Hybridization, IL Press. Dracopoli, N.C. *et al.*, *Current Protocols in Human Genetics*, John Wiley & Sons, Inc.; and Coligan, J.E. *et al.*, *Current Protocols in Protein Science*, John Wiley & Sons, Inc.

Any of a variety of therapeutic genes can be introduced into a cell, in a method of gene therapy, with such a vector. Such genes can be translated into proteins, expressed as antisense nucleic acid sequences or ribozymes, or the like. The genes can be integrated into the host genome, or can be stably maintained or transiently expressed in an unintegrated form.

The types of genes which can be administered are well-known in the art and are disclosed, along with methods of using them for gene therapy, in, *e.g.*, Culver *et al* (1994). *TIG* 10, 1744-178 and U.S. Patents 6,017,896; 5,916,803; 5,871,726; 5,688,773; 5,496,731; 5,631,236; 5,962,424; 5,922,685; 5,789,244; 5,662,896; 5,532,220; 5,888,502; 5,888,814; 5,932,210; and 5,916,803. The genes can be introduced into cells *ex vivo* and reintroduced into a patient, or introduced *in vivo*. Among the general classes of genes which can be administered are: genes which enhance the immunogenicity of a tumor, *e.g.*, genes which encode foreign antigens; genes which initiate apoptosis; genes which enhance immune cells to increase anti-tumor activity, *e.g.*, genes which encode cytokines, such as IL-1, IL-2, IL-4, IL-6, IL-12, TNF- α or β ; GM-CSF, G-CSF, M-CSF, IFN- α , β or γ , TGF- α or β , TNF- α or β , NGF or the like; sensitivity or suicide genes (*e.g.*, genes which encode HSV- or VZV-Tk, which confer sensitivity to gancyclovir; or which encode cytosine deaminase, which confers sensitivity to 5-fluorocytosine; or which encode a non-human purine cleavage enzyme, such as purine nucleoside phosphorylase, which confers sensitivity to a purine substrate which, when cleaved by the enzyme, becomes toxic to the cell); genes which block the expression of oncogenes, *e.g.*, genes which encode antisense K-RAS message); wild type tumor suppressor genes (*e.g.*, p53; p16; retinoblastoma (RB)gene, either full-length p110^{RB} or mutant proteins such as p94^{RB} or p56^{RB}; mitosin; or H-NUC); genes which block the mechanisms by which tumors evade immunological destruction, *e.g.*, the gene which encodes antisense IGF (insulin-like growth factor)-1 message; genes which encode an engineered cytoplasmic variant of a nuclease (*e.g.*, RNase A) or protease (*e.g.* trypsin, papain, proteinase K, carboxypeptidase, etc.); and genes which encode any of the proteinaceous toxins disclosed above.

Many other types of gene therapy are encompassed by the invention and will be evident to one of ordinary skill in the art. For example, any targeting moiety as above can be associated with (e.g., bound to, covalently or noncovalently) a gene of interest and can serve to deliver it to a cell. In one embodiment, the targeting moiety comprises part 5 of a vector which also comprises one or more of: a) a non-viral carrier for the gene to be inserted; b) a fusion protein to enhance the penetration of the vector into the cytoplasm and, optionally, the nucleus, of the cell; and c) a therapeutic gene such as those described above in reference to bacteriophage-mediated gene therapy. Many varieties of components a) and b) will be known to those of skill in the art. For a further discussion 10 of these components, see, e.g., in U.S. Pat. 5,916,803 and the section below regarding the introduction of peptides and similar agents into a cell.

Methods to deliver (administer, introduce) therapeutic agents into a cell, tissue or tumor, or into a patient, *in vitro* or *in vivo*, and to monitor the effects so induced, will be evident to one of ordinary skill in the art. See, e.g., references cited herein, and 15 Vitetta *et al* (1991). *Cancer Res.* 51, 4052. Agents to be administered *in vivo* can be formulated with pharmaceutically acceptable carriers, excipients or vehicles and, in addition, can be supplemented with other medicinal agents such as, e.g., adjuvants, stabilizers, enhancers, or the like.

The specificity of targeting which is engendered by the invention allows for 20 systemic administration. Alternatively, agents can be administered at or near the site of a tissue or tumor to be treated. (Of course, the routes of administration disclosed herein can be employed for methods of detecting (imaging) a target as well as for methods of treatment.). Methods of administration are conventional, and include parenteral and non-parenteral routes of administration. Parenteral routes include, e.g., intravenous, 25 intraarterial, intraportal, intramuscular, subcutaneous, intraperitoneal; intraspinal, intrathecal, intracerebroventricular, intracranial, intrapleural or other routes of injection. Non-parenteral routes include, e.g., oral, nasal, transdermal, pulmonary, rectal, buccal, vaginal, ocular. For non-parenteral delivery, it may be desirable to use agents which enhance transcytosis of a cell-surface receptor/ligand complex, e.g., by administration 30 of brefeldin A or monensin. See, e.g., U.S. patent 5,254,342.

Dosages to be administered can be determined by conventional procedures and, in general, will be known to those of skill in the art. Factors to be considered include the

activity of the specific agent involved, the metabolic stability and length of action of the agent, mode and time of administration, drug combination, rate of excretion, the species being treated, and the age, body weight, general health, sex, diet, and severity of the particular disease-states of the host undergoing therapy. For example, appropriate 5 therapeutic regimens for an immunotoxin (*i.e.*, a conjugate comprising an antibody, or variant or fragment thereof, conjugated to one or more toxin molecules) involve administration to a patient of a dose of between about 0.5 and 2 mg/kg.

A therapeutic agent can be internalized into any of a variety of locations in a cell or tissue. In some cases, it is desirable that a therapeutic agent is internalized into a cell 10 by accompanying Hsp47 as it is recycled into the ER. In other cases, it is desirable that a therapeutic agent is endocytosed into an endosome or lysosome of a cell, *e.g.*, a proteinaceous toxin which must be proteolytically processed in order to become active. In still other cases, it is desirable that the agent evades enzymatic degradation and is transported into the cytoplasm and/or the nucleus. A variety of conventional procedures 15 can be used to enhance the transport of a surface-bound entity into a cell and, in some cases, to avoid such proteolysis. It may be desirable, for example to associate the therapeutic agent, directly or indirectly, with a carrier and/or with a fusion protein, to generate a vector for delivery of the therapeutic agent.

A variety of appropriate carriers (*e.g.*, non-viral carriers) are well-known in the 20 art and are described, *e.g.*, in U.S. Pat. 5,916,803; Cotten *et al* (1993). *Curr. Biol.* 4, 705; Behr (1993). *Acc. Chem. Res.* 26, 274; Felgner (1990). *Adv. Deliv. Rev.* 5, 163; Behr (1994). *Bioconjugate Chem.* 5, 382; and Ledley (1995). *Hum. Gene Ther.* 6, 1129. In a preferred embodiment, the carrier exhibits a long half life in the body, allowing the maximum possible binding of a vector to a target cell. Among the preferred carriers are 25 liposomes or cationic lipids, polypeptides, or proteins, or the like. A carrier can be associated directly (*e.g.*, coupled) or indirectly to a therapeutic agent, by any of a variety of conventional means,

A variety of fusion proteins can enhance the penetration of a vector into a cell, and/or out of an endosome or lysosome and into the cytoplasm of a cell. Many proteins, 30 or fragments or variants thereof, possess fusiogenic properties, including a number of viral proteins. Such proteins will be well known to the skilled worker and are discussed,

e.g., in U.S. Pat. 5,916,803; Hughson (1995). *Current Biol.* 5, 265; Illoekstra (1990). *J. Bioenergetics Biomembranes* 22, 675; and White (1990). *Ann. Rev. Physiol.* 52, 675.

In some cases, a targeting moiety can be modified so that it binds more efficiently to an external domain of Hsp47 and/or is internalized more efficiently into a cell. For example, synthetically produced short peptides, such as some of the 7-mer peptides shown in Table 3, sometimes bind to, but are not taken up by, target cells; whereas the same peptides are internalized when they are displayed at the pIII terminus of a bacteriophage. To enhance the internalization of, e.g., a short synthetic peptide, one can add to the peptide a synthetic peptide linker having desirable properties, e.g., a linker which displays the short peptide in such a way that it can bind to and be internalized by a cell with Hsp47 on its surface, yet does not, itself, bind to cells other than those displaying Hsp47 on the surface; is stable in the circulation; can be processed following internalization, or the like. Other such improvement modifications of target moieties will be evident to those skilled in the art.

In another aspect of the invention, a targeting moiety as above is associated with a detectable moiety that allows the detection (imaging, identification) of a cell which expresses Hsp47 on its surface, e.g., a carcinoma cell, a carcinoma in any stage of development, a tumor, a metastasis (including a micrometastasis), or the like. An agent which comprises a targeting moiety and a detectable moiety is called herein a detectable agent or an imaging agent. Such detection can be accomplished *in vitro* or *in vivo*. Detection methods can be quantitative. For example, the amount of Hsp47 expressed on the surface of a cell, or the number of cells which express Hsp47 on their surface, can be quantitated by exposing the cell, tumor, animal, patient or the like to a detection device which can identify and quantitate the detectable marker.

Detectable moieties encompassed by the invention include, e.g., signal generators [entities which are capable of emitting a detectable amount of energy in the form of electromagnetic radiation (such as X-rays, UV-radiation, IR radiation, visible radiation and the like), and include phosphorescent and fluorescent entities, bioluminescent markers, gamma and X-ray emitters, or the like]; signal reflectors (e.g., paramagnetic entities); or signal absorbers (e.g., electron beam opacifier dyes).

A detectable moiety can be bound to (associated with) a targeting moiety by any of a variety of well-established methods, including those described above for binding

therapeutic moieties to targeting moieties. For example, in methods particularly suited for *in vitro* detection, a radioactive element (e.g., an amino acid) can be incorporated directly into a targeting moiety (e.g., a peptide chain), and detected by autoradiography; or a fluorescent label can be associated with such a targeting moiety via biotin/avidin interaction, association with a fluorescein conjugated antibody, or the like, and detected by immunofluorescence microscopy, flow cytometry (FACscan), or the like.

Target-enhancing metals are encompassed by the invention and are particularly suitable for *in vivo* imaging. Metals according to the invention include, e.g., paramagnetic metals for MRI; heavy metal ions, e.g., with atomic numbers of at least 37, preferably at least 50, for X-ray or ultrasound imaging; and ions of radioactive isotopes for scintigraphy. The choice of metal will be determined by the desired diagnostic (or therapeutic) application. Examples of radioactive ions include, e.g., ions of lanthanides or other metal ions, including isotopes and radioisotopes thereof, such as, e.g., iodine¹²³, iodine¹²⁵, iodine¹³¹, technetium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, copper⁶⁷, yttrium⁹⁰, astatine²¹¹, gallium⁶⁷, iridium¹⁹², cobalt⁶⁰, radium²²⁶, gold¹⁹⁸, cesium¹³⁷, phosphorous³², carbon¹⁴, and tritium ions. Examples of fluorogenic agents include gadolinium and renagraphein.

A metal can be associated with a targeting moiety by any of a variety of means, which are well-known to one of ordinary skill in the art. For methods of making image enhancing moieties, attaching them to targeting moieties, and using them for detection, see, e.g., EP 481,526; GB-A-2169598; and EP 136,812. For example, metals can be bound to (associated with, attached to) targeting moieties by chelating agents, including polychelants, bifunctional polychelants, and salts or macrocyclic intermediates thereof. A variety of methods exist for multiplying the enhancement, e.g., using a compound composed of a backbone molecule, such as a polyamine, to which a plurality of chelating agents are attached.

Routes by which imaging agents can be administered are disclosed above with reference to delivering therapeutic agents. Imaging agents of the invention may be administered to patients for imaging in amounts sufficient to yield the desired contrast with the particular imaging techniques. Generally, dosages of from about 0.001 to 5.0 mmoles of chelated imaging metal ion per kilogram of patient body weight are effective to achieve adequate contrast enhancements. For most MRI applications, preferred

dosages of imaging metal ions will be in the range of about 0.02 to 1.2 mmoles/kg body weight; while for X-ray applications, dosages from about 0.5 to 1.5 mmoles/kg are generally effective to achieve X-ray attenuation. Preferred dosages for most X-ray applications are from 0.8 to 1.2 mmoles of the lanthanide or heavy metal/kg body weight.

5 Another aspect of the invention is a method to screen for an agent which binds specifically to a carcinoma in which Hsp47 is expressed on the surface of at least some of its cells, preferably wherein the agent is useful for treating a carcinoma in a patient or for diagnosing a carcinoma in a patient, comprising identifying an agent which binds specifically to an external domain of Hsp47. Assays for determining if an agent binds to an external domain of Hsp47, and for determining the specificity and/or avidity of the binding, are described elsewhere in this application. Routine procedures are available 10 for screening agents of interest, using such methods.

15 Another aspect of the invention is a kit for treating a patient suffering from a carcinoma in which Hsp47 is expressed on the surface of at least some of the carcinoma cells, or for detecting a carcinoma in which Hsp47 is expressed on the surface of at least some of the carcinoma cells, comprising an agent which binds specifically to an external domain of Hsp47 in an amount effective to generate a cytostatic or cytolytic effect on the carcinoma, or to image the cell above a background of non-carcinoma cells. In a preferred embodiment, the agent 20 comprises, as a targeting moiety, an antibody or a fragment thereof, a peptide, or a bacteriophage on whose surface is a peptide, each of which moieties binds specifically to an external domain of Hsp47. In a most preferred embodiment, the targeting moiety is

25 a monoclonal antibody,

25 a peptide which comprises the consensus motif XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or the consensus motif HyXXXHyHyXXHyXXX (SEQ ID NO: 2), wherein X, independently, can be any amino acid, and Hy, independently, can be any hydrophobic amino acid,

30 a peptide having the sequence of any of SEQ ID NO:3 to SEQ ID NO: 25 of Tables 1 and 2, or

30 a bacteriophage on whose surface is a peptide which binds specifically to an external domain of Hsp47.

5 In one embodiment, the agent comprises, as a therapeutic moiety, a toxin, a radioisotope or radionuclide, an antibody, or a nucleic acid which encodes a therapeutic gene. The invention also encompasses a kit for modulating a cell which expresses Hsp47 on its surface, comprising an agent which binds specifically to an external domain of Hsp47, in an amount effective to modulate the cell. Of course, a kit of the invention will comprise one or more containers for the therapeutic and/or imaging agents.

EXAMPLES

10 Data which are disclosed in the following Examples 1- 6 are presented in the manuscripts: 1) Hebert *et al.* (1999). *J. Cellular Biochemistry* 73, 248-258, which is incorporated by reference in its entirety; and 2) Sauk *et al.* (2000). *J. Cellular Biochemistry*, accepted in January, 2000, which is included herein as Appendix I and is incorporated by reference in its entirety.

15 **Example 1 Expression of Colligin/Hsp47 at the Surface of Oral Epidermoid Carcinoma Cells:**

Studies are performed using established cell lines of human oral squamous cell carcinomas (SCC-4, SCC-9, SCC-15, and SCC-25) and a murine epidermoid cell line, Lewis Lung Carcinoma (LL/2), obtained from ATCC. In addition, a primary gingival fibroblast cell line is used as a control.

20 **A. Flow Cytometric Analysis:**

For these studies, the monoclonal antibody SPA-470 to colligin/Hsp47, (StressGen, Victoria, BC) and a colligin/Hsp47 rabbit polyclonal antibody, prepared against a 22-mer peptide corresponding to the N-terminal sequence of mouse colligin/Hsp47, are used. Monoclonal antibodies for cytometric analyses are directly conjugated with fluorescein using 5(6) carboxyfluorescein-N-hydroxy succinimide ester kit (Boehringer Mannheim, Indianapolis, IN) or labeled with SA-Red670TM following biotinylation of the antibody using EZ-LinkTM Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockville, Ill).

25 Cells grown *in vitro* are washed and incubated in a 0.5% solution of Polyglobin N to block unsaturated Fc receptors and reduce non-specific binding of monoclonal antibodies. Next, 50ml of the cell suspension (1X10⁶ cell/ml) is incubated with 2.5 ml

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(1mg) of antibodies, conjugated with fluorescein, or SA-Red670™ (GibcoBRL, Gaithersburg, MD). After washing, the cell pellet is resuspended in PBS containing BSA for flow cytometric assay. To assess intracellular colligin/Hsp47, cells are first permeabilized with 0.1% Saponin. Samples are then analyzed on a FACScan flow cytometer (Becton Dickinson, San José, CA). The 488 nm Argon laser is run at 15 nW of power. The data from fluorescein conjugates are collected after a 530/30 BP filter. For two-color flow cytometric analysis either fluorescein or Red670™ are employed with propidium iodide. The filters used are 600 nm dichroic SP; 525 ± 15nm BP (fluorescein) and 645 LP (Red670™).

10 Propidium iodide is used to assess cell cycle and stain for dead cells. For these studies a hypotonic citrate solution containing PI is added to ~ 1X10⁶ washed cells to a concentration of 1mM. Cells are labeled for 20 minutes, then analyzed on the FACScan in their staining solution. Orange PI fluorescence is collected after a 585/42 nm BP filter.

15 Electronic compensation is used among fluorescence channels collecting emissions to remove residual spectral overlap. Fluorescence data are displayed on a four-decade long scale. A minimum of 10,000 events is collected on each sample. Analysis of the data is performed with LYSYS II software (Becton Dickinson, Mansfield, MA). Fluorescence dual parameter contour plots are used for exclusion of debris and clumps. This method of gating allows ready discrimination of debris from dead cells (low forward light scatter and high PI fluorescence). The percentage of cells with a G_{1/0}, S, or G₂/M DNA complement is determined from a DNA histogram by region integration using onboard Multicycle® data analysis routines (Phoenix Flow Systems, San Diego, CA).

20 25 Flow-cytometric analysis of SCC cell lines, the LL/2 murine cell line and human gingival fibroblasts reveals that all of the cell lines possess intracellular colligin/Hsp47. Cell cycle analysis further reveals that colligin/Hsp47 expression is not limited to any phase of the cell cycle in either gingival fibroblasts or epidermoid carcinoma cells. However, when cell cycle analysis is performed only for cell surface expression, colligin/Hsp47 is only limited to epidermoid carcinoma cell lines.

30

B. Immunofluorescence Studies: Immunofluorescence microscopy is carried out after the method of Tang *et al.* (Tang *et al.* (1994). *Eur. J. Cell Biology* 65, 298-304:

5 Tang *et al.* (1993). *Eur. J. Cell Biology* 120, 325-328). To visualize cell surface colligin/Hsp47, the cells are not permeabilized but treated and fixed with 1% paraformaldehyde as described for cytometric analyses. The cells are then stained with anti-colligin/Hsp47 antibodies as a primary antibody followed by FITC goat-anti-rabbit or anti-mouse IgG.

Immunofluorescence microscopy of non-permeabilized cells, prepared for flow-cytometric analysis, confirms anti-colligin/Hsp47 staining at the surface.

10 **C. Subcellular Membrane Fractionation:** The method for fractionating plasma membranes is modified after the methods described by Weber *et al.* (1988). "Subcellular distribution of Insulin Receptors" in *Insulin Receptors*. Kahn CR and Harison LS, eds. (New York: Alan R. Liss, Inc), pp. 171-187. In essence, after incubating 5 ml of cells [(2-5) x 10⁶ cells per ml] with or without 1 mM amiloride, the cell suspension is centrifuged at 100 g for 60 s at room temperature. The cell pellets are suspended in 10 ml of tris/EDTA/sucrose buffer (20 mM Tris/HCl, 1mM EDTA and 255 mM sucrose, pH 7.4) at 18-20°C. The pellet is resuspended in 500 µl of Tris/EDTA/sucrose buffer by using a glass-Teflon homogenizer, layered on a 600µl cushion of 1.2 M sucrose in 20 mM Tris/1mM EDTA buffer (pH 7.4), and centrifuged in a Beckman TLS55 rotor at 8,150g at 4°C for 30 min. Plasma membranes collected at the cushion interface are suspended in 2.5 ml of Tris/EDTA /sucrose buffer and centrifuged in a Beckman 20 TLA100.3 rotor at 410,000 g at 4°C for 20 min. The final plasma membrane pellet is resuspended in 60 µl of buffer. The samples are then treated with bacterial collagenase to eliminate the possibility of cytoplasmic derived procollagen-colligin/Hsp47 binding to the cell surface integrin receptors as a result of cell fractionation. The initial supernatant is centrifuged in a Sorvall SS34 rotor at 48,000 g at 4°C for 15 min. and the high-density microsome pellet is resuspended in 40µl of buffer. The supernatant is further centrifuged in a Beckman 70.1 rotor at 300,000 g at 4°C for 75 min. and the low-density microsome pellet is resuspended in 60 µl of buffer.

30 The membrane fractions are characterized by the distribution of 5'-nucleosidase activity, a marker of plasma membrane. Protein is measured with the BCA protein assay kit (Pierce, Rockford, IL). Plasma membranes are directly subjected to PAGE and Western analysis. For Western blots, proteins run on SDS-PAGE are immediately electrotransferred to nitrocellulose paper and blocked with 10% NFDM in 10 mM Tris-

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HCl pH 7.4, 0.9 mM NaCl (TBS) for 2hr and then in TBS/NFDM with 2% NGS (GIBCO, Grand Island, NY). Antiserum or perimmune serum is diluted 1:2000 in the same buffer and incubated with gentle shaking overnight. The nitrocellulose is then rinsed three times for 5 min. in TBS/Tween. Hsp47 is detected with [¹²⁵I]-labeled protein A (New England Nuclear, Boston, MA).

Subcellular membrane fractionation of amiloride and control cells to obtain a plasma membrane fraction, followed by Western blot analysis confirms the presence of colligin/Hsp47 in the plasma membrane 5'-nucleosidase fraction.

Example 2 Binding of Cell Surface Colligin/Hsp47 to $\alpha 1$ (I)-N-propeptide:

10 The cDNA sequences encoding the pro $\alpha 1$ (I) N-propeptide globular domain (NP1) [residues 23-108], and the globular domain + propeptide GlyXaaYaa domain (NP2) [residues 23-151] are prepared as GST-fusion proteins as previously described (Hu *et al.* (1995). *Cellular Biochemistry* 59, 350-367). Fusion protein expression is induced by 0.1 mM IPTG after bacteria reach mid-log phase. The fusion proteins are purified by 15 glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) according the manufacturer's instructions. The proteins are characterized by SDS-PAGE and Western blot analysis as previously described (Hu *et al.*, *ibid*).

NP1 and NP2 affinity beads are prepared, after the method of Hu *et al.* (1995) *ibid*. In essence, GST-fusion proteins are treated with thrombin, dialyzed and the GST protein removed from each reaction mixture by passing it over a column of glutathione-Sepharose. The eluates are collected and lyophilized and coupled to CNBr activated Sepharose. The final beads contain 1-2 mg peptide per 250 μ l. Hsp47 surface binding experiments are carried out by mixing 250 μ l of a 50% (v/v) suspension of peptide-Sepharose (Pharmacia, Piscataway, NJ) with a suspension of plasma membranes from surface-biotinylated SCC cells. After incubation at 4°C for 1h the beads are collected by centrifugation and washed 3 times with an equal volume of Laemmli Buffer. The beads are extracted with 250 μ l Laemmli electrophoresis sample buffer by boiling the sample for 5 min. Following separation by SDS-PAGE, the proteins are transferred to 20 nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma Chemical Co., St. Louis, MO.) using Renaissance Chemiluminescent Reagents (NEN, Cambridge).

To demonstrate the availability of cell surface colligin/Hsp47 to bind procollagen propeptides in living cells, GST-fusion proteins (1 μ g/ml) are added to the culture medium of \sim 1 X10⁶ SCC cells, plated in chambered slides (Nalgene NUNC, Milwaukee, WI), for 10 minutes at 37°C. The cells are then washed in PBS fixed in paraformaldehyde as before, and cell associated fusion proteins (NP1, NP2) are identified with HRPO-conjugated anti-GST antibodies, following the procedures of Sauk *et al.*, (1994). *J. Biol. Chem.* 269, 3941-3946.

Binding experiments performed with biotin labeled plasma membranes and Sepharose bound pro α 1(1) globular domain (residues 23-108) or pro α 1(1) globular domain + propeptide GlyXaaYaa domain (residues 23-151) give similar results. In both instances bands migrating at 46K and 47K are identified. Western blot analysis confirms that both the 46K, unglycosylated colligin/Hsp47, (Hirayoshi *et al.*, 1991) and 47 K bands react with anti-Hsp47 antibodies. These findings are consistent for all of the cell lines.

Immunoperoxide cytochemical staining of GST-fusion proteins bound to cells in culture reveal that cells lines with a higher percentage of cells expressing surface colligin/Hsp47 display a higher percentage of bound GST-NP1 and GST-NP2 fusion proteins.

Example 3 Cell Surface Labeling and Immunoprecipitation:

The methods used were previously described for the characterization of TM4SF complexes with integrins (Berditchevski *et al.*, (1996) *Molec. Biol. of the Cell* 7, 193-207). In essence, cells are labeled with NHS-LC-biotin (Pierce, Rockford, IL) according to kit protocol, and lysed in immunoprecipitation buffer [1% Brij 96, 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 2 mM PMSF, 20 μ g/ml apotinin, and 10 μ g/ml leupeptin]. Immune complexes are collected on protein A beads pre-bound with antibodies, followed by four washes with immunoprecipitation buffer. For more "stringent" conditions, the immunoprecipitation buffer is supplemented with 0.2% SDS. Immune complexes are eluted from protein A beads with Laemmli elution buffer and proteins are separated by SDS-PAGE. Proteins are transferred to nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma Chemical Co., St. Louis MO) using Renaissance Chemiluminescent Reagents (NEN, Cambridge).

Reprecipitations are preformed from Brij 96 lysates prepared from surface-biotinylated SCC cells. After five washes with the immunoprecipitation buffer, the protein complexes are dissociated with 0.5% SDS added to the immunoprecipitation buffer. The eluates are subsequently diluted 1:1 with immunoprecipitation buffer and re-precipitated with the appropriate antibodies directly coupled to Sepharose beads. The samples are then processed as described above.

For cross-linking, cells are treated with DTSSP, a membrane impermeable cross-linker or DSP. After solubilization in immunoprecipitation buffer supplemented with 0.2% SDS protein complexes are immunoprecipitated as above and analyzed under reducing conditions.

Membrane surface proteins are consistently co-precipitated with anti-colligin/Hsp47 antibodies. In cross-linking studies, SCC and LL/2 cells are first pre-treated with a cleavable cross-linker, DSP, then surface labeled with biotin or [¹²⁵I], and subsequent immunoprecipitation is carried out under stringent conditions to disrupt the noncovalent association between Hsp and tetraspanin protein. A colligin/Hsp47-CD9 complex is immunoprecipitated using either anti-CD9 mAbs or anti-colligin/Hsp47 antibodies. A characteristic 47K band is readily detected in all anti-CD9 immunoprecipitates and protein band of ~22K, similar in size to CD9/or CD81, is co-precipitated with anti-colligin/ Hsp47. However, when similar experiments were performed with anti-CD81 mAbs, colligin/Hsp47 is not identified in the immunoprecipitates. However, re-immunoprecipitation of the CD9 immunoprecipitates with anti-colligin/Hsp47 results in a 47 K band in all SCC cells and LL/2 cell lines. To verify whether extracellular regions of Hsp47 and CD9 interact directly, similar cross-linking experiments are performed using DTSSP, a membrane impermeable cross-linker. Treatment of intact SCC and LL/2 cells with either DSP or DTSSP results in co-immunoprecipitation of CD9 and colligin/Hsp47. Treatment with 1mM amiloride greatly enhances the amount of colligin/Hsp47 recovered following immunoprecipitation without altering the profile of proteins precipitated.

Example 4 Tumor Cell Invasion and Phagokinesis:**A: Colloidal Gold Migration Assay:**

Tumor cells are plated on chamber slides precoated with a mixture of 80 μ g/ml type I collagen, 100 μ g/ml Matrigel®, or 100 μ g of laminin-5 and colloidal gold particles and incubated in medium with or without various antibodies. Colloidal gold-coated chamber slides are prepared as described by Albrecht-Buehler ((1977). *Cell* 12, 333-339) with modification for keratinocytes and the inclusion of matrix proteins (Woodley *et al.*, (1988) *J. of Cellular Phys.* 136, 140-146; Kim *et al.*, (1994) *Laboratory Investigation* 71, 401-408; Kim *et al.*, (1994) *J. of Biol. Chem.* 269, 26,926-26,932.) SCC or LL/2 cells are added to each chamber, and 20 minutes latter non-adherent cells are removed and the medium replaced. Cultures are maintained for 24 hr and then fixed in 1X Histochoice (Amresco, Solon, OH) for 1 min, washed in PBS, and dehydrated through graded ethanols. Areas devoid of gold particles identify the phagokinetic tracks. A migration index is determined using image analysis software by measuring the area of phagokinetic tracks associated with cells in random fields under dark field illumination at 100 X (Pilcher *et al.*, 1997) *J. Cell Biol.* 137, 1445-1457. All cells in a field are counted and 25 cells are counted for each experiment. For each experiment, all conditions are done in triplicate.

B: Tumor Cell Invasion Assays:

An *in vitro* assay is modified after that described previously by Chu *et al.* (1993) *Proc. Nat'l. Acad. Sci.* 90, 4261-4265, utilizing Matrigel®, a reconstituted basement membrane. In essence, a modified Boyden chamber containing an 8 μ m-porosity polyvinylpyrrolidone-free polycarbonate filter is precoated with Matrigel® (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA). The lower well of the chamber is then filled with serum free medium containing 500 μ l of 3T6 cell-conditioned medium as a chemoattractant. The upper well is then seeded with 200 μ l of cell suspension at 1.0 $\times 10^4$ cells/chamber plus additives as indicated. The chambers are then incubated at 37°C for 24 hrs. Noninvasive cells are removed from the upper surface of the membrane with a cotton swab and the chamber incubated in 3ml of Dispase (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) for 2 hours and the reaction stopped with 10 mM EDTA. The resulting cells contained in Matrigel®, as well as the cells in the lower chamber, are counted in a Coulter counter. Data are expressed as the percent invasion

through the matrix and membrane relative to the migration through the control membrane. The "Invasion Index" is expressed as the ratio of the percent invasion of a test cell over the percent invasion of a control cell.

Overall treatment and group effects are assessed using an analysis of variance (ANOVA), with post-hoc comparisons based on the Newman-Keuls test ($p \leq 0.05$). The association of mean HSP47 fluorescence labeling with the invasion index is evaluated using the nonparametric Spearman's rank-order correlation coefficient (rho).

Using modified Boyden chambers to assess tumor cell invasion reveals that SCC cells and LL/2 cells show a significant variance in the invasion indices among the cell lines. This variance is associated with the level of colligin/Hsp47 expressed on the cell surface. Chamber assays are then performed where the cells are incubated in the presence of antibodies directed to CD9, CD81 and colligin/Hsp47. Cells incubated in Matrigel® chambers with anti-colligin/Hsp47 result in an increase in the invasion index, while incubation with anti-CD9 or anti-CD81 antibodies are without effect and similar to controls. Treatment with amiloride in each instant dramatically decreases the invasion index in all tumor cell lines. However, amiloride treated cells are unaffected by treatment with anti-colligin/Hsp47 antibodies.

The results of SCC cell migration on colloidal gold assays parallel the results obtained from Boyden chamber assays, but are dependent upon whether the colloidal gold contains Matrigel®, collagen, or laminin-5. The phagokinetic migration index of SCC cells is greatest on laminin-5 followed by collagen and Matrigel®. In particular, the migration tracks are broader and longer on laminin-5 coated colloidal gold than those observed on collagen or Matrigel®. The phagokinetic migration indices on both collagen and Matrigel® matrices are noted to increase following treatment with anti-colligin/Hsp47 antibodies, but are unaffected following treatment with anti-CD9 antibodies. The phagokinetic indices are unaffected following treatment with anti-colligin/Hsp47 on laminin-5 coated surfaces.

Example 5 Identification and Analysis of Hsp47-Binding Peptides:

A. Affinity Panning of a Library of Peptides: Two bacteriophage libraries, with random septapeptide (Ph.D.-7, New England Biolabs; Beverly, MA) or random dodecapeptide (Ph.D.-12, New England Biolabs; Beverly, MA) inserts at the N-terminus

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of pIII protein are used. The Ph.D.-7 library consists of $\sim 2.8 \times 10^9$ electroporated sequences amplified once to yield ~ 70 copies of each sequence in 10 μ l of supplied phage. The Ph.D.-12 library consists of $\sim 2.7 \times 10^9$ electroporated sequences amplified once to yield ~ 55 copies/10 μ l of supplied phage.

5 B. Selection of Hsp47-Binding Bacteriophages by Affinity Panning:

Bacteriophages displaying peptides recognized by Hsp47 are identified using the Ph.D.-7 or Ph.D.-12 kits and protocols modified for a biotinylated target. In essence, 96 wells of a microtitration plate are coated with 15 μ l of streptavidin (1mg/ml) in 135 μ l of 0.1M NaHCO₃, pH 8.6, with gentle agitation in a humidified chamber overnight at 4°C. After removal of the streptavidin solution the wells are washed three times with TBS containing 0.05% Tween 20 (TBS-Tween) and then blocked by incubation for 1 hr at 37°C with 1% BSP in PBS containing 0.1 μ g/ml streptavidin.

15 Next, phage are precomplexed with biotinylated Hsp47 by adding 0.1 µg biotinylated Hsp47 and 2×10^{11} pfu of the input phage in 400 µl TBS-Tween and incubated for 60 min. The phage-target complex solution is then added to the washed blocked plates and incubated at room temperature for 10 min. Biotin is added to a final concentration of 0.1 mM and incubated for 5 min. to displace streptavidin-binding phage. Non-binding phage are discarded and the plates washed 10 x with TBS-Tween (0.1%).
20 The wells are then treated with 15 µl of 1 M Tris-HCl; pH 2.2, containing 1 mg/ml BSA for 5 minutes to elute Hsp47-phage. The samples are neutralized with 1 M Tris-HCl; pH 9.1, and amplified by adding to 20 ml of ER 2537 culture incubated at 37°C with vigorous shaking for 4.5 hrs. The cultures are then transferred to fresh tubes centrifuged at 10,000 rpm, 4°C, for 10 mins. The supernatants are transferred to fresh tubes and phage precipitated with 1/6 volume of PEG/NaCl at 4°C, overnight. To 4 ml of LB media
25 a single colony of ER2537 is inoculated and then incubated at 37°C with vigorous shaking until the culture reaches mid-log phase (O.D.₆₀₀ ~0.5). A pellet is obtained by centrifugation for 15 min at 10,000 rpm, 4°C. The pellet is suspended in 1 ml of TBS and centrifuged for 5 min, 10,000 rpm, 4°C. The supernatant is transferred to a fresh tube and precipitated with 1/6 volume of PEG/NaCl on ice for 1 hr. Following centrifugation the pellet is resuspended in 200 µl TBS. The eluate is titered and plated onto LB/IPTG/XGal plates and incubated overnight. Since the library phage are derived from the common cloning vector M13mp19, which carries the *lacZα* gene, phage plaques appear blue when

plated on media containing Xgal and IPTG. Blue colonies are selected for sequencing or used for a second round of panning. As a control streptavidin is used as a target and after three round of panning to verify a consensus sequence for streptavidin-binding peptides.

C. Screening for Hsp47-Binding Bacteriophages:

5 Clones of blue colonies from plates containing 50-200 colonies are transferred to nitrocellulose filters. The bacteria are washed from the filters with PBS containing 0.05% Tween 20 and 1% bovine serum albumin, and the filters are then incubated for 30 min in the same buffer before washing three times with PBS-Tween. Following incubation for 1hr in 5 ml of biotinylated Hsp47 (0.1-2 μ g/ml in PBS-Tween), the filters
10 are again washed three times in PBS-Tween. The positions of the clones that have secreted Hsp47-binding bacteriophages are then located by one of two methods: the filters are incubated with 5 ml of alkaline phosphatase-conjugated streptavidin (1/10,000 dilution in PBS-Tween; Pierce, Rockford, IL) for 1 hr at room temperature before extensive washing with PBS-Tween, or the filters are incubated for 1 hr in 5 ml of an
15 anti-biotin antibody (1/50,000 dilution in PBS-Tween; Pierce, Rockford, IL), washed, and incubated with a rabbit anti-goat immunoglobulin antibody conjugated to alkaline phosphatase (1/5,000 dilution in PBS-Tween; Pierce, Rockford, IL). In each case, alkaline phosphatase activity is revealed using a mixture of nitroblue tetrazolium/and 5 bromo-4-chloro-3-indolylphosphate tolidium (Bethesda Research Labs, Rockville, MD)
20 as substrate.

D. Determination of the Sequence of Bacteriophage-Displayed Peptides:

Single-stranded bacteriophage DNAs are purified and sequenced as -96 primer an oligonucleotide (5'-CCCTCATAGTTAGCGTAACG-3') (SEQ ID NO: 63). Sequencing reactions are carried out using an ABI Prism Model 373 Version 3.0.

E. Program to Score Hsp47-Binding Peptides:

25 The starting and selected libraries are compared in a position-dependent manner. A statistical analysis is performed by maximum likelihood and bootstrap resampling. This reveals the distribution of residues by position in Hsp47-binding peptides compared to peptides in the original library. To codify the preferences obtained above we use a
30 scoring system previously described for BiP (Cwirla *et al.* (1990) *Proc. Nat'l. Acad. Sci.* 87, 6378-82) to predict Hsp47-binding sites in synthetic and naturally occurring polypeptides. In so doing a score is given to each of the possible 20 amino acids at each

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position of the seven-residue core sequence. These scores are derived from the fold difference in the overall abundance of each residue in the peptides displayed by the selected and nonselected bacteriophage populations. The scoring for each of the seven-residue sequences present in each septapeptide is determined as previously described 5 (Cwirla *et al.*, *ibid*).

To validate the scoring set of Hsp47-binding sequences, a second set of Hsp47-binding sequences not part of the database used to generate the scoring matrix is necessary. To accomplish this a second library consisting of independent recombinant bacteriophages displaying random dodecapeptides is used (Ph.D-12, New England 10 Biolabs, Beverly, MA). Sequences are then compared by position specific iterated BLAST, pattern Hit Initiated BLAST, and BLAST 2 sequences against each other (NCBI). In addition, the hydropathic profiles of two peptides are compared using the Weizmann Institute of Sciences Genome and Bioinformatics database.

E. Analysis of the Hsp47-Binding Peptides:
15 Two bacteriophage libraries with random septapeptide or dodecapeptide inserts at the N-terminus of pIII protein are used; the first to be studied is the septapeptide library. 70 individual bacteriophage clones are picked at random from the unselected library, and the amino acid sequences of the variable septapeptide inserts are deduced from the nucleotide sequences of the corresponding coding region. All amino acids are 20 represented, although their frequencies do not always correspond to those expected from the relative numbers of codons encoding each residue.

The sequences of septapeptides displayed by 54 Hsp47-binding bacteriophages obtained by panning are determined by DNA sequence analysis of the corresponding region of the bacteriophage genome. The peptide sequences from the 54 clones that 25 bound Hsp47 are first considered as a single population and compared with those from the 70 clones picked randomly from the starting library. Tabulation of the hydrophilicities of the Hsp47-binding bacteriophages reveals that two general populations of peptides that are selected by panning. One group of peptides is represented by hydrophilic peptides and the other by a smaller hydrophobic group of 30 peptides.

Comparison of the overall amino acid composition of the two populations, selected and unselected, of septapeptides reveals that asparagine (N), threonine (T),

tyrosine (Y) and proline (P) are particularly enriched, while phenylalanine (F), aspartic acid (D), and arginine (R) are significantly depleted. However, when considered individually, the hydrophobic group of peptides is noticeably enriched in tryptophan, and leucine as well as valine and alanine.

5 The five-residue spacer linking the variable septapeptides to the mature pIII protein contains no enriched residues. Inspection of the selected hydrophobic peptides reveals that a residue motif best described as XHyHyXXXHyHy, where Hy is a large hydrophobic amino acid (usually W,L, or F) and X is any amino acid. This core motif is also identified in selected residue from a Ph.D-12 library represented by a motif 10 XHyHyXXHyXXXXHyHy. The peptide motif described as XXHyHyXXX best describes the selected hydrophilic peptides selected from the library. Interestingly, a similar core motif (HyXXXHyHyXXHyXXX) can also be identified in selected residues from the dodecapeptide library. However, a small number of peptides are completely lacking in hydrophobic residues although they are selected during panning of Hsp47.

15 BLAST program analyses are performed to assess the sequence homology between bacteriophage-displayed peptides and procollagen I and the selected dodecapeptides. Little specific homology is observed based on sequence alone. However, when the hydropathic profile of procollagen I (1) is compared with the dodecapeptides obtained from three rounds of panning, using Kyte-Doolittle method of 20 calculating hydrophilicity over a window length of 7, all of the phage displayed peptides are represented by specific regions within the procollagen molecule. Most of the hydrophobic peptides (see Table 1) are localized to regions within the *N*-propeptide region (residues 59-71) or the *C*-propeptide region (residues 1344-1445). Conversely, most of the hydrophilic peptides (see Table 2) are localized the regions with the helical 25 region of procollagen (residues: 283-295, 470-482, 666-678, 727-739, 1040-1052, and 1087-1099) with only one peptide localized to a sequence within the *N*-propeptide region (residues 100-112).

Example 6 Subcellular localization of Hsp47 binding phage (HBP) on tumor cells:

30 Studies are performed using the cell lines SCC4, SCC9, SCC15 and SCC25 as described above, and a transformed normal oral keratinocyte cell line GMSM-K which

is courtesy of Dr. V. Murrah (UNC, Chapel Hill, NC). Breast carcinomas [HTB126, HTB127] and HTB125 normal breast cells are obtained from ATCC. In addition, prostate cell lines PC-3, LNCaP and PZ-HPV are courtesy of Dr. R. Franklin (UM, Baltimore, MD).

5 A. Flow Cytometric Analysis: Antibodies against Hsp47 are described above, as are methods of conjugating them with fluorescein or Texas Red. Anti-M13 monoclonal antibody is procured from Amersham Pharmacia Biotech (Piscataway, NJ). Cytometric analysis is conducted as described above.

10 25 μ l of HBP are incubated with 10^6 cells (SCC4, SCC9, SCC15, SCC25, HTB126, HTB127, PC-3 and LNCaP) in media for a 1 hr at 37°C. The cells are then washed 3 times with TBS-Tween and the HBP stained with FITC-anti-M13 antibodies and analyzed by flow cytometry. These studies reveal that non-permeabilized tumor cell lines (SCC4, SCC9, SCC15, SCC25, HTB126, and PC-3) possess varying levels of M13 phage staining on their cell surfaces. Furthermore, if the cells are permeabilized prior to the addition of antibody, then cell surface staining coupled with internalized phage reveals enhanced staining. However, GMSM-K, an established epithelial cell line, treated in a like manner, reveals little or no staining. Results similar to those obtained for GMSM-K cells are also obtained for HTB125 and PZ-HPV normal breast and prostate cell lines respectively.

15 20 B. Immunoprecipitation Analysis: To verify the association of M13-binding phage with specific cell lines SCC4 and GMSM-K cells are treated with an HBP for 1 hr, then washed 3 times with TBS-Tween and the plasma membranes are isolated and immunoprecipitated with anti-M13 antibodies. This results in two protein bands one band with a Mr=47k and another band with a Mr=27k. The 47k band is identified as Hsp47 by Western blot analysis.

25 C. Immunofluorescence and Confocal Microscopy:

30 1. Immunofluorescence and confocal microscopy:

 Immunofluorescence microscopy is carried out as described above. To visualize cell surface Hsp47 or M13 bacteriophage, the cells are not permeabilized but treated and fixed with 1-% paraformaldehyde as described for cytometric analyses. However, to prevent non-specific binding, the cells are blocked with 10% pig serum in PBS for 1 hr. The cells are then incubated with anti-Hsp47 or anti-M13 bacteriophage antibodies

(Amersham Pharmacia-Biotech, Piscataway, NJ), washed with PBS, and incubated for 1 h with goat anti-rabbit IgG conjugated with either fluorescein or Texas red. Coverslips are mounted in mounting media containing an antibleaching agent (Kirkegaard & Perry Laboratories, Inc.; Gaithersburg, MD). Cells are examined under a Zeiss II photomicroscope equipped with epifluorescence. Cells untreated with primary antibodies are used as negative controls.

Confocal images are collected using a Zeiss LSM410 confocal microscope. A FT of 488/568 with a barrier filter of 590 is used to detect Texas red staining and a FT of 560 with a barrier at 515-540 is used to generate fluorescein labeled images. Digital images are collected on a ZIP drive and figures generated using Adobe Photoshop 3.0 software (Adobe Systems Inc. Mountain View, CA). No fluorescence is associated with cells after incubation with secondary antibodies alone.

Confocal microscopy shows the fate and co-localization of HBP. These studies reveal that when tumor cell lines are treated with HBP, there is a distribution of staining at the cell surface, in microvesicles, and an intense staining in a perinuclear region that is coincident with the ER. Cells are then treated with M13 bacteriophage followed by double staining with Texas red-anti-Hsp47 antibodies and FITC-M13 antibodies. The localization of Hsp47 is similar to that of M13 phage staining and co-localization of antibodies reveals superimposition, yellow hues, of both M13 staining and Hsp47 staining patterns.

2. Lysosomes and the ER

To label the lysosomal compartment, cells are incubated with 1mg/ml lysine-fixable FITC-dextran (Molecular Probes; Eugene, OR) in growth medium for 4h at 37°C in 5% CO₂. After washing, cells are incubated an additional 30 min to chase the dextran from the early endosomal to the lysosomal compartments. For identification of the early and recycling endosomal compartments, cells are incubated in serum-free medium containing 50 µg/ml FITC-transferrin (Molecular Probes; Eugene, OR) for 30 min at 37°C in 5% CO₂. After treatments to identify the specific subcellular compartments, cells are fixed and processed for immunofluorescence and/or confocal microscopy.

Cells are cultured in the presence of FITC-conjugated dextran, followed by a chase period of 30 min to remove the dextran from early endosomal compartments, before fixation and immunostaining with the Texas red conjugated anti-M13 antibodies.

SSC4 cells, which are representative of the other cell lines, demonstrates a clear identification of FITC-dextran to vesicular structures, however, the HBP staining is primarily limited to punctate vesicles in the cytoplasm and a perinuclear zone. To verify that HBP is not significantly targeted to lysosomes, SCC4 cells are fed latex beads and HBP and then fixed and processed by for immunofluorescence using anti-M13 antibodies. In SCC4 cells, M13 signal cannot be located at the periphery of the bead, suggesting that there is minimal association with the phagosomes.

To label endosomes, SCC4 and GMSM-K cells are cultured in the presence of FITC-conjugated or Texas red-conjugated transferrin before fixation and staining with anti-M13 antibodies. Analysis by confocal microscopy indicates that transferrin stains both the plasma membrane (ring staining at the edge of cells) and recycling endosomes (subcellular punctate staining). A very similar and overlapping pattern is observed for M13 antibody staining, consequently superimposition of the two images indicates colocalization (yellow hues) of the two signals at the punctate subcellular region and plasma membrane. Noteworthy is that GMSM-K cells provide like patterns of staining with conjugated dextran and transferrin but are not stained by anti-M13 antibodies.

D. Subcellular Fractionation

Plasma membranes are fractionated as described above.

The membrane fractions are characterized by the distribution of 5'-nucleosidase activity, a marker of plasma membrane. Plasma membranes are directly subjected to PAGE and Western analysis. For Western blots, proteins run on SDS-PAGE are immediately electrotransferred to nitrocellulose paper and blocked with 10% non-fat dry milk (NFDM). Antiserum or perimmune serum is diluted 1:2000 in the same buffer and incubated with gentle shaking overnight. The nitrocellulose is then rinsed three times for 5 min. in TBS/Tween. Antibodies to Hsp47 and M13 bacteriophage are detected with [¹²⁵I]-labeled protein A (New England Nuclear, Boston, Mass) or Western blot analysis.

E. Peptide Synthesis

Setapeptides and dodecapeptides are prepared by continuos flow solid-phase synthesis and analyzed by high-pressure liquid chromatography and mass spectrophotometry as described in previously (Cwirla *et al.*, *ibid*).

Example 7 Determination of the efficacy of Hsp47-binding peptides and Hsp47 monoclonal antibodies in homing chemotherapeutic drugs to tumor sites in oral squamous carcinoma xenografts.

A. Effect of Doxorubicin Conjugated Hsp47 Tumor-Homing Peptides and Hsp47 Monoclonal Antibodies on Tumor Progression: Doxorubicin is used as a model compound to show that the homing of drug to cell surface Hsp47 in well-differentiated tumors effectively reduces the dose of drug required for a tumor response, or increases the effectiveness of drug on the chemotherapeutic index in a human oral squamous cell carcinoma xenograft model.

B. Hsp47 monoclonal antibody doxorubicin conjugates: Doxorubicin immunoconjugates are formulated using a malonate linker. In essence, monoclonal antibody is conjugated to BAMME-CH DMB linker in 0.5 M borate buffer in the presence of dimethylformamide. After chromatographic purification, the functional antibody is deblocked by the addition of 100 mM carbohydrazide and purified chromatographically. Finally, the deblocked functional antibody is reacted with 10-mM doxorubicin and purified chromatographically. Similar immunoconjugates have been shown to maintain the immunoreactivity of the antibody and to have equivalent potency to unconjugated free doxorubicin without manifesting toxicity as measured by weight loss and deaths in a nude mouse xenograft mode.

C. Hsp47 binding protein doxorubicin conjugates: Hsp47 peptides are synthesized by the UM Biopolymer Laboratory Core Laboratory and purified by high-performance liquid chromatography. The peptides are conjugated to doxorubicin (Aldrich) with 10ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC, Sigma) and N-hydroxysuccinimide (NHS, Sigma). The conjugates are then freed of reactants by gel filtration on Sephadex G25. The presence of free drug is monitored by HPLC and nuclear magnetic resonance to be <5% of the preparation.

D. Pharmacokinetics of doxorubicin distribution as free drug, peptide conjugate, and monoclonal antibody conjugate:

1. Animals and Drug Administration: Specific-pathogen-free, adult mice (5-6 weeks of age) are maintained under conventional housing conditions. Intravenous bolus doses of drug, free doxorubicin, peptide-conjugate doxorubicin, and monoclonal antibody conjugated doxorubicin, are administered through a lateral tail vein.

2. Sampling: Blood is sampled at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 960, and 1440 min after dosing. In addition, hearts, lungs, livers, kidneys, spleens, skeletal muscles, and tumors are collected at the same times notes for blood samples. In each study, blood and tissues from mice killed 5 min after delivery of vehicle serve as controls. Blood is collected by cardiac puncture into heparinized syringes, transferred to Eppendorf microcentrifuge tubes and stored on ice until centrifuged at 13,000 x g for 5 min to obtain plasma. Tissues are rapidly dissected, kept on ice until weighed, and then snap frozen in liquid nitrogen. Plasma, tissues, and dosing solutions are stored frozen at -70°C until analysis.

10 3. Analysis of Doxorubicin: Plasma and tissue concentrations of doxorubicin are determined by high performance liquid chromatography (HPLC). Plasma samples are extracted by conventional procedures. Two hundred μ l samples of plasma are mixed with internal standard (4'-epodoxorubicin) and then mixed vigorously with 0.6 ml of chloroform/2-propanol (1:1, v/v) for 15 sec. Ammonium Sulfate (approximately 0.5 g) is added to the resulting gel and mixed thoroughly. The triphasic mixture is centrifuged for 15 min at 13,000 x g. The resulting upper phase is transferred to a conical test tube and dried unde a N₂ jet. The dried residue is redissolved in 100 μ l of mobile phase, transferred to autosampler vials, and 75 μ l is injected into the HPLC system described below.

15 20 Tissue samples are thawed, immediately transferred to 17 x 100 mm polypropylene tubes that are held in an ice bath, and homogenized, using a Polytron (Brinkman Instruments, Westbury, NY), in parts (weight to volume) of phosphate-buffered saline (1.2mM KH₂PO₄, 2.9 mM Na₂HPO₄, 154mM NaCl, pH 7.4, Biofluids, Inc., Rockville, MD). One part of each homogenate is then mixed with internal standard, extracted with chloroform/2-propanol, and prepared for injection into the HPLC system.

25 The HPLC system used includes a Hewlett-Packard (Palo Alto, CA) model 1100 autosampler and a Waters (Milford, MA) M45 pump. The column employed is an ALLTECH (Deerfield, IL) 10 μ m C₁₈ Econosil column (250 length, 4.6 mm i.d.). The mobile phase consists of acetonitrile: 0.32 M ortho-phosphoric acid (27:43, v/v) and is pumped at 1 ml/min. Column eluent is monitored with an Aminco fluoromonitor set at excitation wavelength of 470 nm and emission wavelength >500 nm. With this method, good separation of doxorubicin, doxorubicinol, and internal standard is achieved, with

retention times of 12.5, 7, and 15 min, respectively. The lower limit of quantitation is 0.1 nm. The detector signal is processed with a Hewlett-Packard 3392A integrator so as to integrate the area under each peak. Doxorubicin concentration in each sample is calculated by determining the ratio of doxorubicin peak area to that of the corresponding internal standard peak and comparing that ratio to a concomitantly performed standard curve prepared in the appropriate matrix.

4. Pharmacokinetic Analysis: Time courses of plasma concentrations of doxorubicin versus time are analyzed by both non-compartmental and compartmental methods. Area under the curve from zero to infinity (AUC) and terminal half-life ($t_{1/2}$) are estimated by non-compartmental analysis with the LaGrange function as 10 implemented by the LAGRAN computer program. CL_{tb} is calculated from the definition:

$$CL_{tb} = \text{Dose}/\text{AUC}$$

and steady-state volume of distribution (V_{DSS}) is calculated from the formula:

$$V_{DSS} = CL_{tb}/\text{kel.}$$

15 In addition, individual concentrations of doxorubicin in plasma versus time are fit to compartmental models with the program ADAPT II, which uses a Nelder Mead simplex as the algorithm. Two- and three-compartment, open, linear models are fit to the data. Model discrimination is based on Akaike's Information Criteria (AIC), defined as:

$$AIC - 2p + n(\ln WSSR),$$

20 where p represents the number of parameters in the model, n equals the number of observations, and WSSR represents the weighted sum of squares residuals.

25 E. Treatment of mice bearing tumors with Hsp47 homing drugs: Mice with size-matched tumors ($\sim 1 \text{ cm}^3$) are randomized into six treatment groups of no less than six animals per group: vehicle only, free doxorubicin, doxorubicin-Hsp47 binding peptide, doxorubicin-control nonbinding peptide, doxorubicin-monoclonal conjugate, Hsp47 monoclonal antibody. Power analyses indicate that at least 6 animals per group are required to see a 30% difference in survival. Generally, doxorubicin dosage in nude mice with human xenographs is 50 to 200 $\mu\text{g}/\text{week}$. Since the homing of drug to tumor is more effective than free drug, initial studies are performed with dosages of 5, 10 and 30 15 $\mu\text{g}/\text{week}$. The concentration of doxorubicin as equivalents is adjusted by measuring the absorbency of drug and conjugates at 490 nm and a calibration curve established to ascertain equivalents for peptide and monoclonal conjugates. The fraction of surviving

5 animals is determined over a course of 40 days and plotted as a Kaplan-Meier survival curve. Survival is determined by examining animals daily to determine mortality. Next, a dose escalating experiment is performed in which mice are treated with doxorubicin-Hsp47 binding peptide and doxorubicin-conjugated Hsp47 monoclonal antibodies at a dose of 30 μ g of doxorubicin equivalent every 21 days for 84 days. A Kaplan-Meier survival curve is constructed. In addition, following necropsy, the primary tumor weight and size are ascertained, and the presence of lymph node and pulmonary metastasis is determined. Lymph node weight and lung weight are also measured as a gross measure of metastasis supported by histological assessment.

10 To assess toxicity, drug dosage is escalated to 200 μ g of doxorubicin-equivalent per mouse. Mice receiving elevated doses of drug receive a single dose of free doxorubicin, doxorubicin-Hsp47 binding peptide, or doxorubicin-Hsp47 monoclonal antibody. Animals are then followed for 14 days and the mean fraction of animals depicted in a Kaplan-Meier survival curve.

15 Animals treated with tumor homing doxorubicin-conjugates survive longer an at lower equivalent doses of drug than animals receiving free drug. In addition, along with a greater survival is a reduction in tumor size as weight, and lymph node and drug metastasis. If unconjugated Hsp47 monoclonal antibody itself has an immunotherapeutic effect in mediating a response against tumor, this is evident only in the C57BL model, 20 as immunodeficient nude mice do not respond in such a manner and the use of unconjugated controls.

25 From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

30 The entire disclosure of all applications, patents and publications, cited above and in the figures are hereby incorporated by reference.

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APPENDIX I

BINDING MOTIFS OF CPB2 A POTENTIAL CELL
SURFACE TARGET FOR CARCINOMA CELLS

Previously we have shown (*J. Cellular Biochem.*, 1999) that among many cell lines the *CPB2* gene product, Hsp47, eludes its retention receptor, *erd2P*, resulting in the appearance of Hsp47 on the cell surface associated with the tetraspanin protein CD9. In that Hsp47 possesses a highly restricted binding cleft, random peptide display libraries were used to characterize peptides binding to Hsp47 and then to target this protein on carcinoma cell lines *in vitro*.

Comparison of the clones obtained from panning revealed little specific homology based on sequence alone. To determine whether carcinoma cells expressing Hsp47 could selectively take up the selected bacteriophages, traditional immunofluorescence and confocal microscopy were employed. These studies revealed that phage-displaying Hsp47 binding peptides bound to cell lines expressing Hsp47 and that the peptides were rapidly taken up to a location coincident with Hsp47 staining. These observations were confirmed by cytometric analyses. These data indicate that *CPB2* product may provide a molecular target for chemotherapy and/or imaging of malignancies.

Abbreviations: *CPB2* , collagen binding protein 2; ER, endoplasmic reticulum; ATCC, American Type Culture Collection; TBS, Tris buffered saline; PBS, phosphate buffered saline; PEG, polyethylene glycol, FITC, fluorescein isothiocyanate.

There is an on going quest to identify specific cell surface targets by which to direct treatment or to image neoplastic cells. For example, one group of proteins that has been used as targets for drug delivery is chimeric molecules created by cancer-associated chromosomal abnormalities. However, such molecules are usually unique to a particular tumor (Baron et al., 1997; Diez de Medina SG et al., 1997). In addition to the aforementioned proteins, potential sources of such targets are the products of genes that are amplified at a particular chromosome locus in malignancy. For example the chromosome locus 11q13-14 is commonly amplified in human cancers that include cancers of the head and neck, lung, esophagus, bladder and breast (Schuuring et al., 1992). This amplicon is large, spanning 2.5-5 Mb and harbors several genes with known oncogenic potential (Bekri, 1997). In breast cancer, this locus is amplified in ~13% of primary tumors while in head and neck cancers amplification may represent 40%-76% of tumors. A more detailed map of this region has evolved indicating that as many as five distinct amplification units exist on 11q13. Among these units is a genomic area encompassing the GARP gene at 11q13.5-q14.1. Assessment of this region, located telomeric to *CCDN1* and *EMS1*, has revealed a number of genes in the regional map, namely *CBP2* (*Hsp47*) and "spot 14" at chromosome locus 11q13.5 with, *CLNS1A*, *UVRAG*, and *PAK1* located telomeric to this later region. Thus, narrowing the core of the 11q13-q14 amplicon to a 350-kb area encompassing D11S533 on its telomeric side (Bekri, 1997; Moncur, 1998).

In assessing this amplicon it became evident that the gene product of

CBP2 was associated with, and may distinguish a group of malignant tumors (Morino et al., 1997; Morino et al., 1995; Morino et al., 1994; Shirakami et al., 1995a; Shirakami et al., 1995b; Shirakami et al., 1995), as well as, being localized to extravillous cytotrophoblasts and decidual cells at the fetal maternal interface (Pak et al., 1997; Shirakami et al., 1995b; Morino et al., 1995; Morino et al., 1994; Morino et al., 1997; Shirakami et al., 1995). Interestingly, *CBP2*, mapped to chromosome 11q13.5, shares a locus with *Spot 14* a key gene expressed in lipogenic neoplasms (Moncur, 1998).

Normally, the *CBP2* gene product, Hsp47, is limited to the ER-Golgi where it is first associated with procollagen chains at a very early point during translation of nascent chains (Sauk et al., 1994). Hsp47 is retained within these cellular compartments by recycling of the *erd2* gene product, KDEL receptor, that associates with the COOH-terminus sequence RDEL of Hsp47 (Sauk et al., 1997). However, in some tumors Hsp47 is expressed independent of its chaperone properties and eludes or leaks from this surveillance mechanism and manifests on the cell surface, but is not secreted into the medium. Although previous studies have demonstrated that Hsps such as gp96 are tightly surface-bound peripheral membrane proteins, the precise mechanism of anchorage was unclear, albeit ionic interactions with other proteins could be excluded (Tamura Y, 1997). Hsp47, like gp96, lacks sequence characteristics for farnesylation, palmitation, isoprenylation or myristylation; consequently covalent bonding also appears to be an unlikely anchoring mechanism (Tamura Y, 1997; Altmeyer et al., 1996; Tamura Y, 1997). Interestingly, Hsp47 has been shown to associates

with the tetraspanin protein CD9 in some epidermoid carcinoma cell lines (Hebert et al., 1999). Moreover, in these instances Hsp47 has been shown to be easily recovered, even without the use of cross-linking, from membrane immunoprecipitates utilizing anti-CD9 antibodies, suggesting the presence of hydrophobic interactions between these proteins (Hebert et al., 1999).

Hsp47 unlike many other chaperones has been shown to possess a limited number of intracellular ligands (Nakai et al., 1989). Although previous studies have defined the Hsp47 binding to a region defined by the anti-propeptide antibody SP1.D8 (Hu et al., 1995), to a region of procollagen to N-propeptides of the $\alpha 1(I)$ -chains between residues 23-151, and to gelatin (Nagata et al., 1988), specific peptide motifs have yet to be determined for this binding (Hu et al., 1995).

Here, we report on a repertoire of bacteriophage-peptides obtained from panning experiments with Hsp47. Colocalization of some of these peptides with Hsp47 in a number of tumor cell lines demonstrates that the peptides can be directed to an intracellular location spatially coincident with Hsp47. These results indicate that cell surface Hsp47 is not permanently anchored to the cell surface and that this protein undergoes recycling with an intracellular pool. In addition, these studies suggest that expression of the CBP2 in tumors may provide a target for phage directed gene therapy, a mechanisms by which to deliver drugs, or a means of imaging occult disease and metastases.

MATERIAL & METHODS

DEPARTMENT OF DEFENSE

Cell Lines: Studies were performed using a number of established cell lines of human oral squamous cell carcinomas [SCC4, SCC9, SCC15, and SCC25] were obtained from ATCC, and a transformed normal oral keratinocyte cell line GMSM-K was courtesy of Dr. V. Murrah (UNC, Chapel Hill, NC). Breast carcinomas [HTB126, HTB127], and HTB125 normal breast cells were likewise obtained from ATCC. In addition, prostate cell lines PC-3, LNCaP, and PZ-HPV were courtesy of Dr. R. Franklin (UM, Baltimore, MD). In the studies presented here, cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium containing 10% fetal calf serum, hydrocortisone (0.4 μ g/ml, Sigma) at 37°C in a 5% CO₂ air atmosphere. For breast carcinoma cell lines 10 μ g/ml of insulin was added to the medium as prescribed by ATCC and PZ-HPV cells were grown in KSF medium. Cells were subcultured by disaggregation with trypsin (0.1%)-EDTA (0.01%) in phosphate buffered saline [PBS] at pH 7.5.

Antibodies: The monoclonal antibody SPA-470 to Hsp47 (StressGen, Victoria, BC) was used, as well as, a Hsp47 rabbit polyclonal antibody prepared against a 22-mer peptide corresponding to the N-terminal sequence of mouse Hsp47 that was conjugated to Keyhole Limpet hemocyanin (Sauk et al., 1994). The anti-M13 monoclonal antibody was procured from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibodies for cytometric analyses were directly conjugated with fluorescein using 5(6) carboxyfluorescein-N-hydroxy succinimide ester kit (Boehringer Mannheim, Indianapolis, IN) or labeled with SA-Red670TM following biotinylation of the antibody using EZ-LinkTM Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockville, IL).

Affinity Panning of a Library of Peptides: To study the binding specificity of Hsp47, we utilized two bacteriophage libraries with random septapeptide Ph.D.-7, New England Biolabs; Beverly, MA) or dodecapeptide (Ph.D.-12, New England Biolabs; Beverly, MA) inserts at the N-terminus of pIII protein. The Ph.D.-7 library consists of $\sim 2.8 \times 10^9$ electroporated sequences amplified once to yield ~ 70 copies of each sequence in 10 μ l of supplied phage. The Ph.D.-12 library consisted of $\sim 2.7 \times 10^9$ electroporated sequences amplified once to yield ~ 55 copies/10 μ l of supplied phage.

Selection of Hsp47-Binding Bacteriophages by Affinity Panning:

Bacteriophages displaying peptides recognized by Hsp47 were identified using the Ph.D.-7 or Ph.D.-12 kits (New England Biolabs; Beverly, MA) and the protocols modified for a biotinylated target. In essence, 96 wells of a microtitration plate were coated with 15 μ l of streptavidin (1mg/ml) in 135 μ l of 0.1M NaHC0₃ pH 8.6, with gentle agitation in a humidified chamber overnight at 4°C . After removal of the streptavidin solution the wells were washed three times with TBS containing 0.05% Tween 20 (TBS-Tween) and then blocked by incubation for 1 hr at 37°C with 1% BSP in PBS containing 0.1 μ g/ml streptavidin.

Next, phage was precomplexed with biotinylated Hsp47 by adding 0.1 μ g biotinylated Hsp47 and 2×10^{11} pfu of the input phage in 400 μ l TBS-Tween and incubated for 60 min. The phage-target complex solution was then added to the washed blocked plates and incubated at room temperature for 10 min. Biotin was added to a final concentration of 0.1 mM and incubated for 5 min. to displace

streptavidin-binding phage. Non-binding phage were discarded and the plates washed 10 x with TBS-Tween (0.1%). The wells were then treated with 15 μ l of 1 M Tris-HCl; pH 2.2, containing 1 mg/ml BSA for 5 minutes to elute Hsp47-phage. The samples were neutralized with 1 M Tris-HCl; pH 9.1, and amplified by adding to 20 ml of ER 2537 culture incubated at 37°C with vigorous shaking for 4.5 hrs. The cultures were then transferred to fresh tubes centrifuged at 10,000 rpm, 4°C, for 10 mins. The supernatants were transferred to fresh tubes and phage precipitated with 1/6 volume of PEG/NaCl at 4°C, overnight. To 4 ml of LB media a single colony of ER2537 was inoculated and then incubated at 37°C with vigorous shaking until the culture reached mid-log phase (O.D.₆₀₀ ~0.5). A pellet was obtained by centrifugation for 15 min at 10,000 rpm, 4°C. The pellet was suspended in 1 ml of TBS and centrifuged for 5 min, 10,000 rpm, 4°C. The supernatant was transferred to a fresh tube and precipitated with 1/6 volume of PEG/NaCl on ice for 1 hr. Following centrifugation the pellet was resuspended in 200 μ l TBS. The eluate was titered and plated onto LB/IPTG/XGal plates and incubated overnight. Since the library phage are derived from the common cloning vector M13mp19, which carries the *lacZ α* gene, phage plaques appear blue when plated on media containing Xgal and IPTG. Blue colonies were selected for sequencing or used for a second round of panning. As a control streptavidin was used as a target and after three round of panning a to verify a consensus sequence for streptavidin-binding peptides.

Screening for Hsp47-Binding Bacteriophages:

Clones of blue colonies from plates containing 50-200 colonies were transferred to nitrocellulose filters. The bacteria were washed from the filters with PBS containing 0.05% Tween 20 and 1% bovine serum albumin, and the filters were then incubated for 30 min in the same buffer before washing three times with PBS-Tween. Following incubation for 1hr in 5 ml of biotinylated Hsp47 (0.1-2 μ g/ml in PBS-Tween), the filters were again washed three times in PBS-Tween. The positions of the clones that had secreted Hsp47-binding bacteriophages were then located by one of two methods: the filters were incubated with 5 ml of alkaline phosphatase-conjugated streptavidin (1/10,000 dilution in PBS-Tween; Pierce, Rockford, IL) for 1 hr at room temperature before extensive washing with PBS-Tween, or the filters were incubated for 1 hr in 5 ml of an anti-biotin antibody (1/50,000 dilution in PBS-Tween; Pierce, Rockford, IL), washed, and incubated with a rabbit anti-goat immunoglobulin antibody conjugated to alkaline phosphatase (1/5,000 dilution in PBS-Tween; Pierce, Rockford, IL). In each case, alkaline phosphatase activity was revealed using a mixture of nitroblue tetrazolium/and 5 bromo-4-chloro-3-indolylphosphate toliidium (Bethesda Research Labs, Rockville, MD) as substrate.

Determination of the Sequence of Bacteriophage-Displayed Peptides:

Single-stranded bacteriophage DNAs were purified and sequenced as -96 primer an oligonucleotide (5'-CCCTCATAGTTAGCGTAACG-3'). Sequencing reactions were carried out using an ABI Prism Model 373 Version 3.0.

Program to Score Hsp47-Binding Peptides:

To characterize peptides that bind to Hsp47 we compared the starting and

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selected libraries in a position-dependent manner. A statistical analysis was performed by maximum likelihood and bootstrap resampling. This revealed the distribution of residues by position in Hsp47-binding peptides compared to peptides in the original library. To codify the preferences obtained above we used a scoring system previously described for BiP (Cwirla et al., 1990) that would be used to predict Hsp47-binding sites in synthetic and naturally occurring polypeptides. In so doing a score is given to each of the possible 20 amino acids at each position of the seven-residue core sequence. These scores were derived from the fold difference in the overall abundance of each residue in the peptides displayed by the selected and nonselected bacteriophage populations. The scoring for each of the seven residue sequences present in each septapeptide was determined as previously described (Cwirla et al., 1990).

To validate our scoring set of Hsp47-binding sequences, a second set of Hsp47-binding sequences not part of the database used to generate the scoring matrix is necessary. To accomplish this a second library consisting of independent recombinant bacteriophages displaying random dodecapeptides was used (Ph.D-12, New England Biolabs, Beverly, MA). Sequences were then compared by position specific iterated BLAST, pattern Hit Initiated BLAST, and BLAST 2 sequences against each other (NCBI). In addition, the hydropathic profiles of two peptides were compared using the Weizmann Institute of Sciences Genome and Bioinformatics database.

Peptide Synthesis:

Septapeptides and dodecapeptides were prepared by continuos flow solid-phase

synthesis and analyzed by high-pressure liquid chromatography and mass spectrophotometry as described in previously (Cwirla et al., 1990).

Cytometric Analyses:

Cells grown *in vitro*, as described above, were washed and incubated in a 0.5% solution of Polyglobin N to block unsaturated Fc receptors and reduce non-specific binding of monoclonal antibodies (Takeshita et al., 1995). Next, 50 ml of the cell suspension (1×10^6 cell/ml) was incubated with 2.5 ml (1mg) of antibodies, conjugated with fluorescein, or SA-Red670TM (GibcoBRL, Gaithersburg, MD). After washing, the cell pellet was resuspended in PBS containing BSA for flow cytometric assay. To assess intracellular Hsp47 and M13 bacteriophage, cells were first permeabilized with 0.1% Saponin as previously described (Tang et al., 1994; Tang et al., 1993). Samples were then analyzed on a FACScan flow cytometer (Becton Dickinson, San José, CA). The 488 nm Argon laser was run at 15 nW of power. The data from fluorescein conjugates were collected after a 530/30 BP filter. For two-color flow cytometric analysis either fluorescein or Red670TM were employed with propidium iodide. The filters used were 600 nm dichroic SP; 525 \pm 15nm BP (fluorescein) and 645 LP (Red670TM).

Propidium iodide was used to assess cell cycle and stain for dead cells. For these studies a hypotonic citrate solution containing PI was added to $\sim 1 \times 10^6$ washed cells to a concentration of 1mM. Cells were labeled for 20 minutes, then analyzed on the FACScan in their staining solution. Orange PI fluorescence was collected after a 585/42 nm BP filter.

Electronic compensation was used among fluorescence channels collecting emissions to remove residual spectral overlap. Fluorescence data were displayed on a four-decade long scale. A minimum of 10,000 events was collected on each sample. Analysis of the data was performed with LYSYS II software (Becton Dickinson, Mansfield, MA). Fluorescence dual parameter contour plots were used for exclusion of debris and clumps. This method of gating allowed ready discrimination of debris from dead cells (low forward light scatter and high PI fluorescence).

Immunofluorescence and Confocal Microscopy: Immunofluorescence microscopy was carried out after the method of Tang et al, (Tang et al., 1994; Tang et al., 1993). To visualize cell surface Hsp47 or M13 bacteriophage, the cells were not permeabilized but treated and fixed with 1-% paraformaldehyde as described for cytometric analyses. However, to prevent non-specific binding, the cells were blocked with 10% pig serum in PBS for 1 hr. The cells were then incubated with anti-Hsp47 or anti-M13 bacteriophage antibodies (Amersham Pharmacia-Biotech, Piscataway, NJ), washed with PBS, and incubated for 1 h with goat anti-rabbit IgG conjugated with either fluorescein or Texas red. Coverslips were mounted in mounting media containing an antibleaching agent (Kirkegaard & Perry Laboratories, Inc.; Gaithersburg, MD). Cells were examined under a Zeiss II photomicroscope equipped with epifluorescence. Cells untreated with primary antibodies were used as negative controls.

Confocal images were collected using a Zeiss LSM410 confocal microscope. A FT of 488/568 with a barrier filter of 590 was used to detect Texas

red staining and a FT of 560 with a barrier at 515-540 were used to generate fluorescein labeled images. Digital images were collected on a ZIP drive and figures generated using Adobe Photoshop 3.0 software (Adobe Systems Inc. Mountain View, CA). No fluorescence was associated with cells after incubation with secondary antibodies alone.

To label the lysosomal compartment, cells were incubated with 1mg/ml lysine-fixable FITC-dextran (Molecular Probes; Eugene, OR) in growth medium for 4h at 37°C in 5% CO₂. After washing, cells were incubated an additional 30 min to chase the dextran from the early endosomal to the lysosomal compartments. For identification of the early and recycling endosomal compartments, cells were incubated in serum-free medium containing 50 µg/ml FITC-transferrin (Molecular Probes; Eugene, OR) for 30 min at 37°C in 5% CO₂. After treatments to identify the specific subcellular compartments, cells were fixed and processed for immunofluorescence and/or confocal microscopy.

Subcellular Fractionation of Plasma Membranes: The method for fractionating plasma membranes was modified after the methods described by Weber et al. (Weber TM et al., 1988). The samples were treated with bacterial collagenase to eliminate the possibility of cytoplasmic derived procollagen-Hsp47 binding to the cell surface integrin receptors as a result of cell fractionation. The initial supernatant was centrifuged in a Sorvall SS34 rotor at 48,000 g at 4°C for 15 min. and the high-density microsome pellet was resuspended in 40 µl of buffer. The supernatant was further centrifuged in a Beckman 70.1 rotor at 300,000 g at 4°C for 75 min. and the low-density microsome pellet was

resuspended in 60 μ l of buffer.

The membrane fractions were characterized by the distribution of 5'-nucleosidase activity, a marker of plasma membrane (Avruch and Wallach, 1971). Plasma membranes were directly subjected to PAGE and Western analysis. For Western blots, proteins run on SDS-PAGE were immediately electrotransferred to nitrocellulose paper and blocked with 10% non-fat dry milk (NFDM). Antiserum or perimmune serum was diluted 1:2000 in the same buffer and incubated with gentle shaking overnight. The nitrocellulose was then rinsed three times for 5 min. in TBS/Tween. Antibodies to Hsp47 and M13 bacteriophage were detected with [¹²⁵I]-labeled protein A (New England Nuclear, Boston, Mass) or Western blot analysis as described previously (Sauk et al., 1997)

RESULTS

To elucidate the binding motifs of Hsp47, we utilized two bacteriophage libraries with random septapeptide or dodecapeptide inserts at the N-terminus of pIII protein. Because peptides containing at least seven or eight residues are generally required for efficient binding we first chose a library of bacteriophages that displayed septameric peptides (Ph.D.-7, New England Biolabs; Beverly, MA). To begin to characterize these peptides, 70 individual bacteriophage clones were picked at random from the unselected library, and the amino acid sequences of the variable septapeptide inserts were deduced from the nucleotide sequences of the corresponding coding region. All amino acids are represented, although their frequencies do not always correspond to those expected from the relative numbers of codons encoding each residue.

The sequences of septapeptides displayed by 54 Hsp47-binding bacteriophages obtained by panning were determined by DNA sequence analysis of the corresponding region of the bacteriophage genome. The peptide sequences from the 54 clones that bound Hsp47 were first considered as a single population and compared with those from the 70 clones picked randomly from the starting library. Tabulation of the hydrophilicities of the Hsp47-binding bacteriophages revealed that two general populations of peptides that were selected by panning. One group of peptides was represented by hydrophilic peptides and the other by a smaller hydrophobic group of peptides (Figure 1).

Comparison of the overall amino acid composition of the two populations, selected and unselected, of septapeptides revealed that asparagine (N),

threonine (T), tyrosine (Y) and proline (P) were particularly enriched, while phenylalanine (F), aspartic acid (D), and arginine (R) were significantly depleted (Figure 2a, 2b). However, when considered individually, the hydrophobic group of peptides was noticeably enriched in tryptophan, and leucine as well as valine and alanine.

The five-residue spacer linking the variable septapeptides to the mature pIII protein contained no enriched residues. Thus, indicating that the spacer residues are unlikely to contribute to the binding activity of selected bacteriophages, allowing us to look only within the variable septapeptide sequences for the presence of a binding motif. Inspection of the selected hydrophobic peptides revealed that a residue motif best described as XHyHyXXXHyHy, where Hy is a large hydrophobic amino acid (usually W,L, or F) and X is any amino acid. This core motif is also identified in selected residue from a Ph.D-12 library represented by a motif XHyHyXXHyXXXXHyHy. Taken together these data suggest that the peptide-binding site contain a deep hydrophobic pocket separated by charged hydrophilic residues (Figure 3). The peptide motif described as XXHyHyXXX best described the selected hydrophilic peptides selected from the library. Interestingly, a similar core motif (HyXXXHyHyXXHyXXX) could also be identified in selected resides from the dodecapeptide library. However, a small number of peptides were completely lacking in hydrophobic residues although they were selected during panning of Hsp47.

Recognizing that procollagen I was a natural ligand for Hsp47, BLAST

program analyses were performed to assess the sequence homology between bacteriophage-displayed peptides and procollagen I and the selected dodecapeptides. Interestingly, little specific homology was observed based on sequence alone. However, when the hydropathic profile of procollagen I (1) was compared with the dodecapeptides obtained from three rounds of panning, using Kyte-Doolittle method of calculating hydrophilicity over a window length of 7, all of the phage displayed peptides were represented by specific regions within the procollagen molecule. Interestingly, most of the hydrophobic peptides were localized to regions within the *N*-propeptide region (residues 59-71) or the *C*-propeptide region (residues 1344-1445). Conversely, the most of the hydrophilic peptides were localized the regions with the helical region of procollagen (residues: 283-295, 470-482, 666-678, 727-739, 1040-1052, and 1087-1099) with only one peptide localized to a sequence within the *N*-propeptide region (residues 100-112) (figure 4).

To gain insight into the subcellular localization of Hsp47-binding phage (HBP) on tumor cells, we incubated 25 μ l of HBP with 10⁶ cells (SCC4, SCC9, SCC15, SCC25, HTB126, HTB127, PC-3 and LNCaP) in media for a 1 hr at 37°C. The cells were then washed 3 times with TBS-Tween and the HBP stained with FITC-anti-M13 antibodies and analyzed by flow cytometry. These studies revealed that non-permeabilized tumor cell lines (SCC4, SCC9, SCC15, SCC25, HTB126, and PC-3) possessed varying levels of M13 phage staining on their cell surfaces. Furthermore, if the cells were permeabilized prior to the addition of antibody, then cell surface staining coupled with internalized phage

revealed enhanced staining. However, GMSM-K, an established epithelial cell line, treated in a like manner, revealed little or no staining. Figure 5 depicts a representative tumor cell line, SCC4 cells, compared to GMSM-K cells. Results similar to those obtained for GMSM-K cells were also obtained for HTB125 and PZ-HPV normal breast and prostate cell lines respectively.

To verify the association of M13-binding phage with specific cell lines SCC4 and GMSM-K cells were treated with an HBP for 1 hr, then washed 3 times with TBS-Tween and the plasma membranes were isolated and immunoprecipitated with anti-M13 antibodies. This resulted in two protein bands one band with a Mr=47k and another band with a Mr=27k (Figure 6). The 47k band was identified as Hsp47 by Western blot analysis.

Confocal microscopy was also employed to determine the fate and co-localization of HBP. These studies revealed that when tumor cell lines were treated with HBP that there was a distribution of staining at the cell surface, in microvesicles and an intense staining in a perinuclear region that was coincident with the ER (Hebert et al., 1999). Cells were then treated with M13 bacteriophage followed by double staining with Texas red-anti-Hsp47 antibodies and FITC-M13 antibodies. The localization of Hsp47 was similar to that of M13 phage staining and co-localization of antibodies revealed superimposition, yellow hues, of both M13 staining and Hsp47 staining patterns (Figure 7).

Next, we determined whether HBP localized to the late endosomal/lysosomal compartment. To label the lysosomal compartment, cells were cultured in the presence of FITC-conjugated dextran, followed by a chase

period of 30 min to remove the dextran from early endosomal compartments, before fixation and immunostaining with the Texas red conjugated anti-M13 antibodies. SCC4 cells, which were representative of the other cell lines, demonstrated a clear identification of FITC-dextran to vesicular structures, however, the HBP staining was primarily limited to punctate vesicles in the cytoplasm and a perinuclear zone (Figure 8). To verify that HBP was not significantly targeted to lysosomes SCC4 cells were feed latex beads and HBP and then fixed and processed by for immunofluorescence using anti-M13 antibodies. In SCC4 cells, M13 signal could not be located at the periphery of the bead, suggesting that there was minimal association with the phagosomes.

Since we had previously shown that Hsp47 was expressed on the cell surface we considered whether HBP bound to cell surface Hsp47 might be present in recycling endosomes. To label these compartments SCC4 and GMSM-K cells were cultured in the presence of FITC-conjugated or Texas red-conjugated transferrin before fixation and staining with anti-M13 antibodies. Analysis by confocal microscopy indicated that transferrin stained both the plasma membrane (ring staining at the edge of cells) and recycling endosomes (subcellular punctate staining). A very similar and overlapping pattern was observed for M13 antibody staining, consequently superimposition of the two images indicated colocalization (yellow hues) of the two signals at the punctate subcellular region and plasma membrane (Figure 9). Noteworthy was that GMSM-K cells provided like patterns of staining with conjugated dextran and transferrin but were not stained by anti-M13 antibodies (not shown).

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Based on the sequence of peptides isolated by panning, septapeptides and dodecapeptides consistent with the predicted models obtained from scoring were synthesized, labeled with FITC and incubated with SCC cells that were shown to bind and take-up HBP. Although none of the cell lines took up the septapeptides all of the synthesized dodecapeptides could be noted in the cytoplasm of tumor cells after 15 min. of incubation (Figure 10).

DISCUSSION:

The use of random peptide libraries has been shown to be a valuable tool for identifying novel therapeutic molecules. The libraries represent an enormous number of peptide sequences displayed either on the virion surface of filamentous phage clones or on a solid phase synthetic support. Central to this strategy is the observation that peptides isolated by affinity selection from such libraries typically interact with biologically relevant domains of the target proteins (Smith and Scott, 1993; Kitamura et al., 1992; Burkhardt et al., 1991; Smith et al., 1995; Pasqualini et al., 1996; Pasqualini and Ruoslahti, 1996; Ruoslahti, 1997; Ruoslahti, 1996; Hutchcroft et al., 1992; Borst et al., 1993). This strategy often results in a number of peptides that apparently bind to a single domain of a protein or receptor. Curiously, the peptides often lack sequence similarity and are thus reminiscent of the discovery of mimotopes (Bowditch et al., 1996; Partidos et al., 1997; Bowditch et al., 1996), where short peptides bind to the antigen-binding site of antibodies even though they differ in sequence from the

antigen. Clearly, this appears to be the case here, where myriads of peptides were localized from both libraries. These peptides, however, could be divided into two groups. One group characterized by a prevalence of hydrophobic residues and a second group characterized by a preponderance of charged hydrophilic residues. The enrichment of valine and alanine as well as tryptophan and leucine in peptides displayed by this selected group of bactiophages is consistent with the affinity of Hsp47 to a region that is determined by its hydrophobic character. In addition, the demonstration of HBP binding by dodecapeptides with a motif of XXXXXHyXXHyXHyHy indicates that the hydrophobic domains of the ligand need not be located at the pIII terminus. Thus, our data suggests that these selected peptides describe a peptide binding site for Hsp47 that contains at a long pocket which can accommodate the side chains of large hydrophobic and aromatic amino acids and that contain adjacent regions that can interact with charged residues. These conclusions are supported by three-dimensional structural modeling studies which reveal that mature Hsp47 possesses a binding region as a long, deep cleft, which at physiologic pH, the base is formed by a β -sheet with sides formed by helices. The helices project hydrophobic amino acid residue side chains in toward the cleft while β -sheet project hydrophobic amino acid residue side chains up from the bottom. (Davids et al., 1995). Moreover, this model explains the ability of Hsp47 to bind to hydrophobic regions within the *N*-propeptide as described previously (Hu et al., 1995) and likewise to the backbone of denatured collagen (gelatin) (Nagata et al., 1988). Interesting, was the discovery that regions within

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the C-propeptide region of procollagen might also provide a binding site for Hsp47, since carboxyterminal association of procollagen chains are implied in chain assembly (Chessler, 1993b; Lees, 1997; Oliver, 1996; Lees, 1994; Chessler, 1993a).

To gain further understanding into the nature and fate of Hsp47 displayed on the cell surface of cancer cells we localized M13-phage selected from the bacteriophage libraries to the cell surface of a number of oral cancer, breast and prostate cell lines utilizing flow cytometric analyses. In so doing, we demonstrated that permeabilization of the cells prior to antibody staining enhanced the FACS signaling, suggesting that M13 was internalized into the cells. Immunoprecipitation of the plasma membranes of the tumor cells exposed to selected bacteriophages displaying Hsp47 binding peptides revealed that Hsp47 was immunoprecipitated with the M13 bacteriophage as well as another 27kD protein which may represent CD9 (Hebert et al., 1999), thus, substantiating that the selected HBP were associated with cell surface Hsp47 complexes. Confocal imaging was then employed to begin to localize the fate of the internalized selected peptide displaying bacteriophage. These studies revealed that short-term incubation resulted in selected peptides being localized to the cell surface and recycling endosomes. This was not unexpected since membrane turnover of cell surface proteins and receptors normally follow such a course. However, surprisingly only a small amount of the internalized peptides could be identified in lysosomes distinguished by FITC-dextran staining. Antithetically, when the cells were stained for both M13 displayed HBP and Hsp47 there was a

concurrent localization, yellow hues, of both antibodies to coincident intracellular locations represented by recirculating endosomes and the ER.

Collectively, these data indicate that unlike cell surface peptide hormone receptors that undergo internalization by endocytosis upon binding to ligands, which are then sorted in endosomes to lysosomes where they are presumably degraded (Gruenheid, 1999). Cell surface ER proteins are routed with their ligands back to the ER with only a small amount of the ligand destined for lysosomal processing. Conceivably, the use of an ER protein with a binding capacity to a restricted number of ligands will permit targeting of drug conjugates or imaging compounds directly to tumor cells expressing these proteins. Such targeting offers the possibility of minimizing nonselective toxic effects. Moreover, one would expect that multidrug resistance would be less important in the case of cell surface ER-mediated transport of the drug across the cell membrane (Czerwinski et al., 1998). Although these *in vitro* data indicate that a cell surface expressed Hsp47 contain elements that might be able to deliver a very selective tumor agent, elements that may have importance in determining this activity have yet to be tested in this model. These elements include the type of toxic moiety which could deliver its effect in the ER and the necessary level of Hsp47 expressed on the cell surface to deliver drug and the ability to recycle for additional ligand binding and transport (Czerwinski et al., 1998). In that septapeptides displayed at the pIII terminus of bacteriophage were taken up by tumor cells while their synthetic homologues were not internalized, indicates that the use of such peptides would necessitate peptide linkers , that are stable in the

circulation, capable of displaying the peptides for binding and are yet able to be processed following internalization. Ongoing research in our laboratory is directed to resolving these issues and to the application of our findings to clinically relevant tumors.

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FIGURE LEGENDS**Figure 1. Distribution of Hydophobicity Scores Computed for Peptides
Displayed by Bacteriophages Selected from the PhD-7 Library Following
Panning Against Hsp47.**

The distribution of overall hydrophobicity scores calculated for each peptide using the hydropathy scale of Kyte and Doolittle (1982) are shown for 54 peptides displayed by bacteriophages obtained from Hsp47 binding following three rounds of panning.

**Figure 2. Relative Abundance of the 20 Amino Acids In Septapeptides
Displayed by the Library of PhD-7 Bacteriophages.**

Single stranded DNA was purified from 70 bacteriophage clones from the PhD library, and the sequences of the septapeptides displayed by these

bacteriophages were deduced from the DNA sequence of the corresponding region of the bacteriophage genome. The figure shows the frequency of occurrence of each amino acid calculated as the number observed divided by the total number of residues in the 70 septapeptides. The amino acids are grouped according to the number of codons that specify them, and the frequency expected for each group if all codons were utilized with equal efficiency is shown by open bars (Cwirla et al., 1990) . (B) The overall amino acid composition of 54 septapeptides from the Hsp47-binding PhD-7 bacteriophages was compared with that of 70 septapeptides from clones picked randomly from the PhD-7 starting library. The fold increases or fold decrease in the abundance is shown.

Figure 3: Schematic Model of the Peptide-Binding Site of Hsp47.

The peptide backbone is shown as an extended chain. The side chains of two adjacent residues extend into deep pockets in the peptide-binding site that have overall preferences for large hydrophobic or aromatic side chains. The data in figure 3 indicate that amino acids at position 2&3 in septa peptides and 2&3 and/or 11&12 make favorable contacts with Hsp47 side chains. The hydrophobic pockets are flanked by regions that containing charged residues at position 4,5,6 in septapeptides and positions 4 &5 and 7,8,9 & 10 in dodecapeptides.

Figure 4: The Hydropathic Profile of Procollagen I Compared with Selected

Peptides from PhD-12 Library.

Using the Kyte Doolittle method of calculating hydrophilicity (1982) over a window length of 7, selected phage from the PhD-12 library were compared with procollagen I alpha chains. The residues indicated in the boxes above the procollagen I profile indicate the residues similar to Hsp47 binding peptides selected from the PhD-12 library after three rounds of panning. Arrows at the C-propeptide regions indicate amino acids critical for the association and assembly of procollagen (Chessler, 1993b; Lees, 1997; Oliver, 1996; Lees, 1994; Chessler, 1993a).

Figure 5: FITC-antiphage M13 Staining of GMSM-K (Control) and SCC-4 Cells.

Panel **a** and **b** represent control GMSM-K cells and SCC-4 cells respectively depicting membrane staining. Panels **c** and **d** represent GMSM-K cells and SCC-4 cells respectively, in which the cells have been permeabilized to demonstrate intracellular staining. The areas within the boxes represent FITC staining for phage-peptide.

Figure 6: Immunoprecipitation of Membrane Proteins by Anti-Phage M13 Antibodies.

Bands represent anti-M13 phage binding proteins immunoprecipitated as described in *Methods*. Lanes **a-f** represent M13 clones selected from PhD-7 and PhD-12 libraries. Lanes **a-c** depict PhD-7 clone 3,5,&7 respectively and lanes **d-f**

depict PhD-12 clone 2,5,&9 respectively.

Figure 7: Confocal Microscopic Images of SCC-4 cells Stained with Anti-Hsp47 and Anti-M13 antibodies.

Panel **a** depicts tumor cell stained with FITC conjugated anti-M13 antibodies, panel **b** depicts Texas red conjugated anti-Hsp47 antibodies, and panel **c** depicts spatial co-localization, yellow hues, of FITC and Texas red staining.

Figure 8: Confocal Microscopic Images of SCC-4 cells Stained with FITC-Dextran and Texas red-Anti-M13 antibodies.

Panel **a** depicts tumor cell stained with FITC conjugated Dextran, panel **b** depicts Texas red conjugated anti-M13 antibodies, and panel **c** depicts spatial co-localization of FITC and Texas red staining. Only a minor portion of anti-M13 antibodies co-localizes with FITC-Dextran, yellow hues.

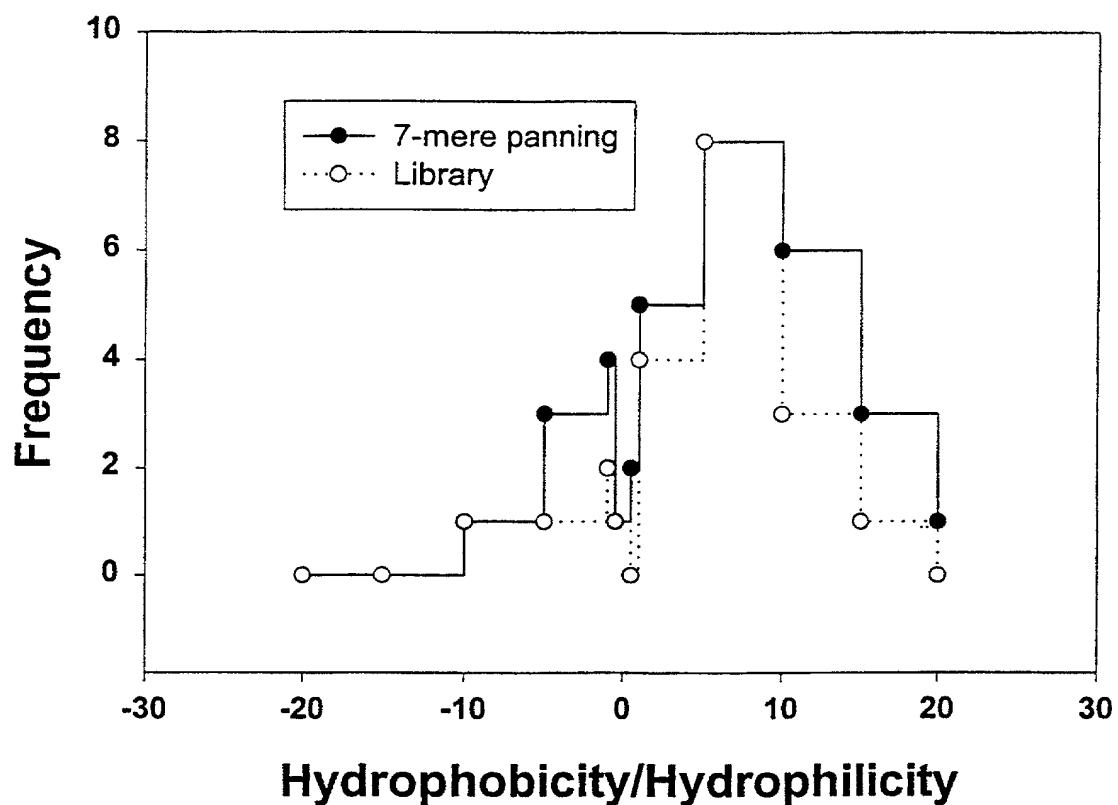
Figure 9: Confocal Microscopic Images of SCC-4 cells Stained with FITC-Transferrin and Texas red-Anti-M13 antibodies.

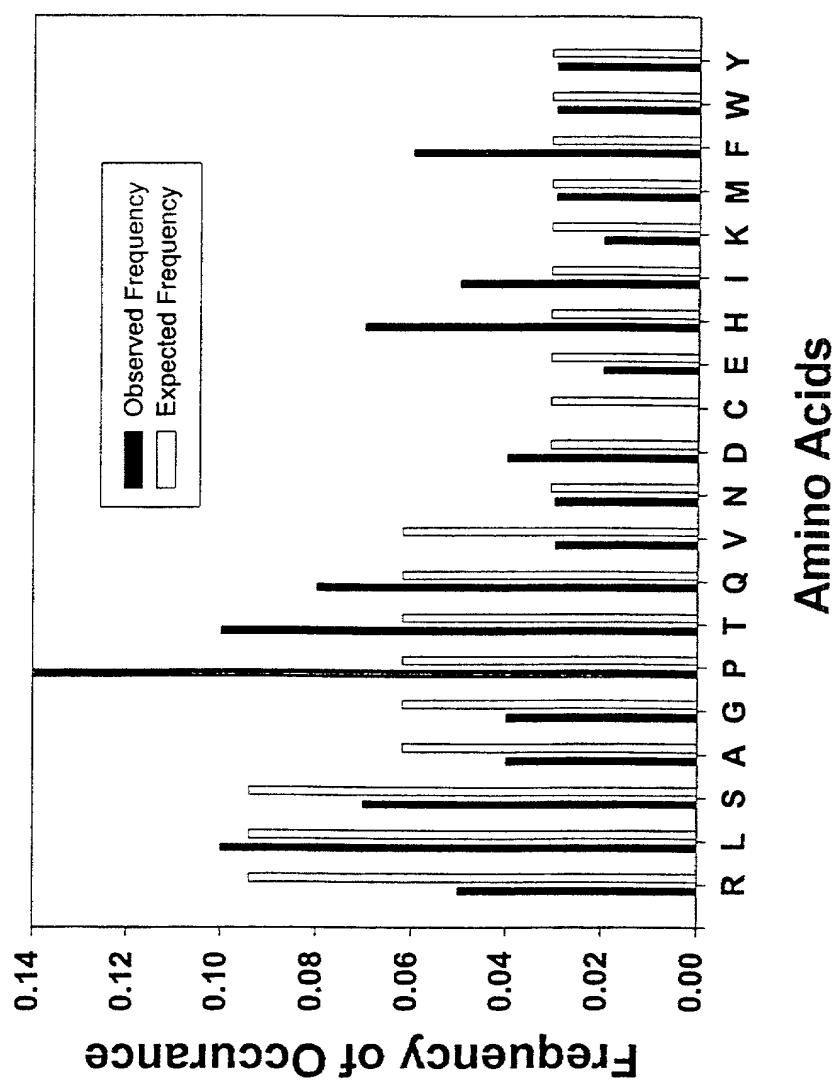
Panel **a** depicts tumor cell stained with FITC conjugated Transferrin, panel **b** depicts Texas red conjugated anti-M13 antibodies, and panel **c** depicts spatial co-localization, yellow hues, of FITC and Texas red staining.

Figure 10: Fluorescein isothiocyanate Conjugated Dodecapeptide Uptake in SCC Cells. Dodecapeptide corresponding to a cloned peptide was synthesized

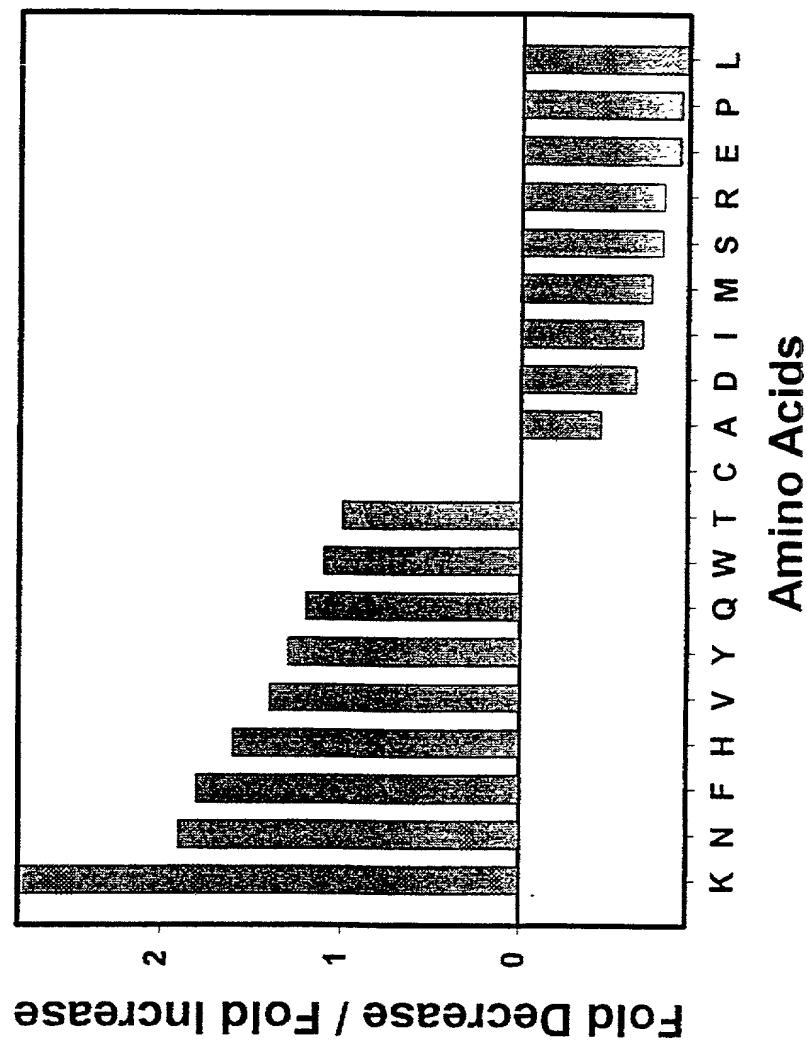
- 73 -

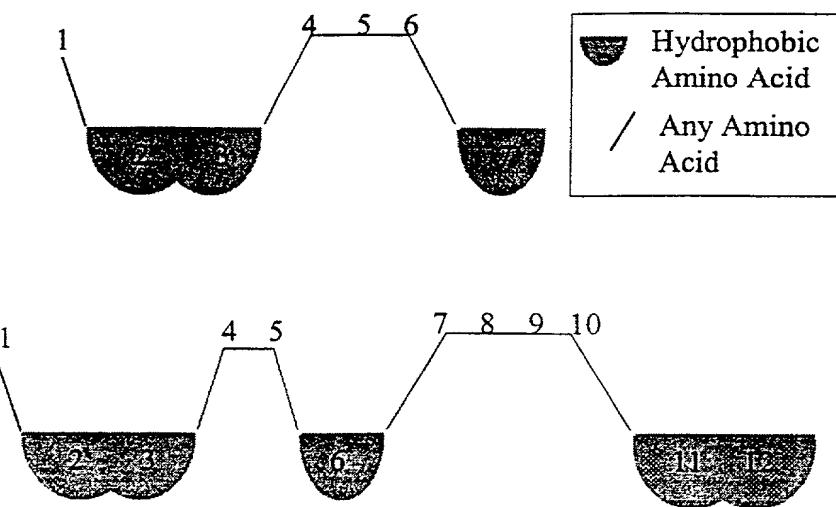
and conjugated to fluorescein isothiocyanate as described in *Methods*. The peptide was then incubated with SCC-4 cells for 30 min., washed and processed for fluorescence microscopy. The green fluorescence depicted about the nuclear area and in punctate staining represent cellular distribution of the peptide uptake.

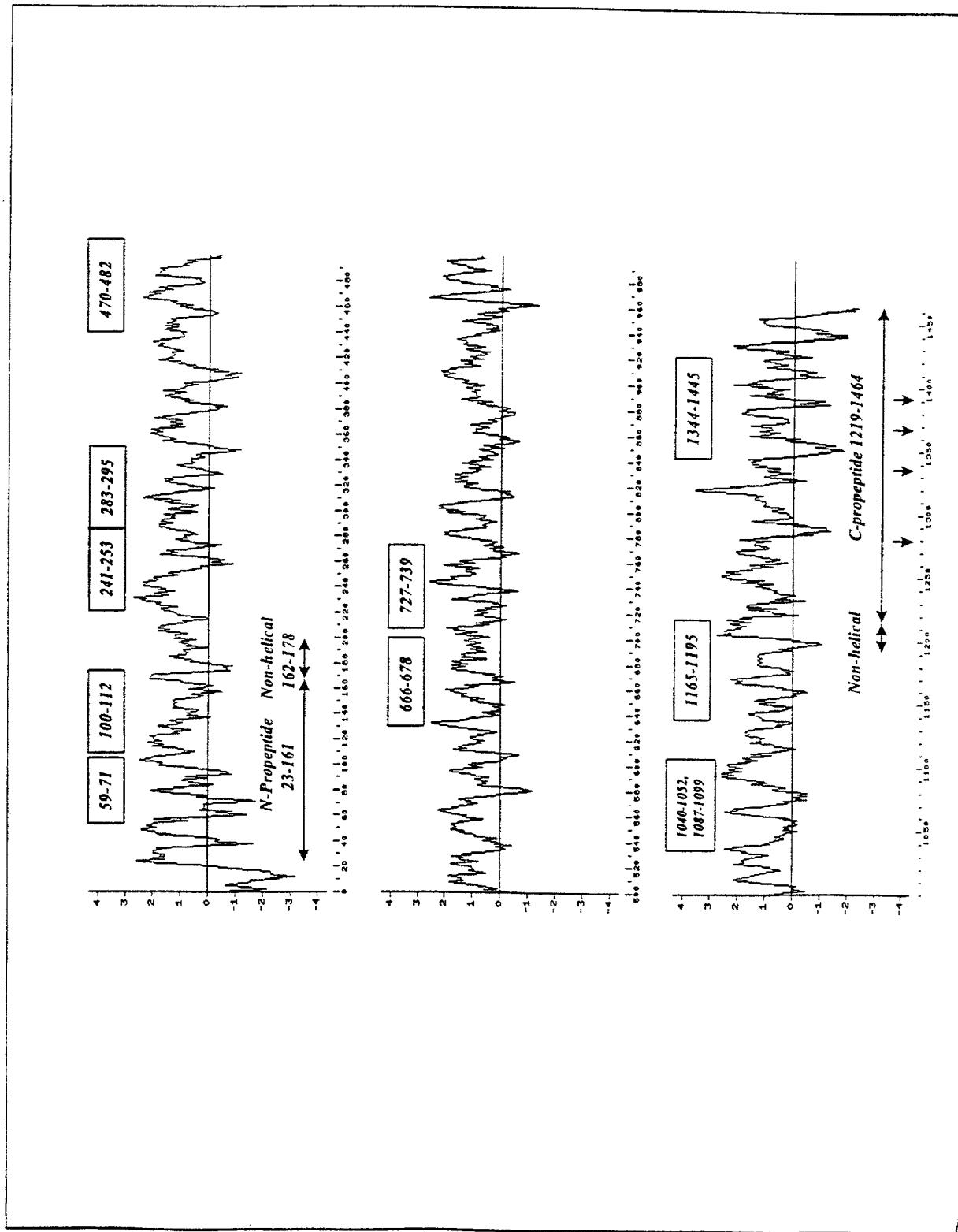




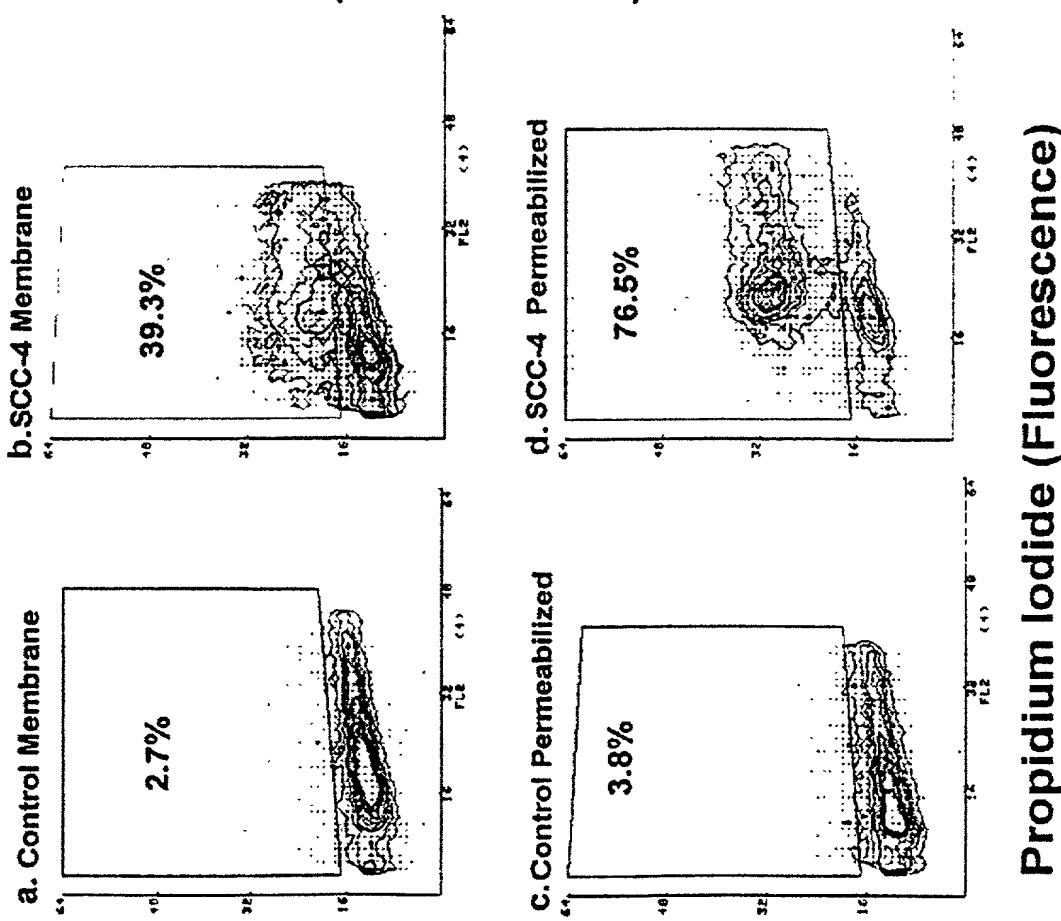
-76-







FITC (Fluorescence)



What is claimed is:

1. A method for treating a patient suffering from a carcinoma in which Hsp47 is expressed on the surface of at least some of cells, comprising administering to the patient an effective amount of an agent comprising a targeting moiety which binds specifically to an external domain of Hsp47.
- 5
2. The method of claim 1, wherein the targeting moiety is an antibody, or a fragment thereof.
3. The method of claim 2, wherein the antibody is a monoclonal antibody.
4. The method of claim 1, wherein the targeting moiety is a peptide.
- 10 5. The method of claim 4, wherein the peptide is XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or HyXXXHyHyXXHyXXX (SEQ ID NO: 2), wherein X, independently, can be any amino acid, and Hy, independently, can be any hydrophobic amino acid.
6. The method of claim 4, wherein the peptide is one of SEQ ID NO:3 to SEQ ID NO: 25 of Tables 1 and 2.
- 15 7. The method of claim 1, wherein the targeting moiety is a bacteriophage on whose surface is a peptide which binds specifically to an external domain of Hsp47.
8. The method of claim 1, wherein the agent further comprises a therapeutic moiety which is a toxin, a radioisotope or radionuclide, an antibody, or a nucleic acid which encodes a therapeutic gene.
- 20 9. The method of claim 1, for modulating the interaction of a tumor cell with an intracellular matrix, tumor cell invasion, migration or motility of malignant cells, or tumor cell metastasis.

10. A method for modulating a cell which expresses Hsp47 on its surface, comprising administering to the cell an effective amount of an agent comprising a targeting moiety which binds to an external domain of Hsp47.

11. The method of claim 1, wherein the Hsp47 is human.

5 12. A method for detecting a carcinoma in which Hsp47 is expressed on the surface of at least some cells, comprising contacting the carcinoma with a detectable agent comprising a targeting moiety which binds specifically to an external domain of Hsp47.

10 13. The method of claim 12, wherein the targeting moiety is an antibody or a fragment thereof, a peptide, or a bacteriophage on whose surface is a peptide, each of which moieties binds specifically to an external domain of Hsp47.

14. The method of claim 12, wherein the detectable agent is detectable by MRI, X-Ray, gamma scintigraphy, or CT scanning.

15 15. A method for detecting a cell which expresses Hsp47 on its surface, comprising administering to the cell a detectable agent comprising a targeting moiety which binds specifically to an external domain of Hsp47.

16. The method of claim 12, wherein the Hsp47 is human.

17. A method to screen for an agent which binds specifically to a carcinoma in which Hsp47 is expressed on the surface of at least some cells, comprising identifying an agent comprising a targeting moiety which binds specifically to an external domain of Hsp47.

20 18. The method of claim 17, wherein the agent is useful for treating a carcinoma in a patient.

19. The method of claim 17, wherein the agent is useful for diagnosing a carcinoma in a patient.

20. A kit for treating a patient suffering from a carcinoma in which Hsp47 is expressed on the surface of at least some cells, or for detecting a carcinoma in which Hsp47 is expressed on the surface of at least some cells, comprising an agent comprising a targeting moiety which binds specifically to an external domain of Hsp47 in an amount effective to generate a cytostatic or cytolytic effect on the carcinoma, or to image the cell above a background of non-carcinoma cells.

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21. The kit of claim 20, wherein the targeting moiety is an antibody or a fragment thereof, a peptide, or a bacteriophage on whose surface is a peptide, each of which moieties binds specifically to an external domain of Hsp47.

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22. The kit of claim 20, wherein the agent further comprises a therapeutic moiety, which is a toxin, a radioisotope or radionuclide, an antibody, or a nucleic acid which encodes a therapeutic gene.

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23. A kit for modulating a cell which expresses Hsp47 on its surface, comprising an agent comprising a targeting moiety which binds specifically to an external domain of Hsp47, in an amount effective to modulate an activity of the cell.

24. A peptide which binds specifically to an external domain of Hsp47 expressed on the surface of a cell, comprising XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or HyXXXHyHyXXHyXXX (SEQ ID NO: 2),
wherein X, independently, can be any amino acid, and Hy, independently, can be any hydrophobic amino acid, and
wherein the binding is effective to modulate the cell..

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25. A peptide which binds specifically to an external domain of Hsp47 expressed on the surface of a carcinoma, comprising XHyHyXXHyXXXXHyHy (SEQ ID NO: 1) or HyXXXHyHyXXHyXXX (SEQ ID NO: 2),
wherein X, independently, can be any amino acid, and Hy, independently, can be any hydrophobic amino acid, and

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wherein the binding is effective to generate a cytostatic or cytolytic effect on the carcinoma or to image the carcinoma above a background of non-carcinoma cells.

26. A peptide of claim 24, wherein said peptide is not full-length collagen, and is not naturally occurring collagen or a fragment thereof.

5 27. The peptide: SEQ ID NOS: 3- 13 of Table 1.

28. The peptide: SEQ ID NOS: 14-25 of Table 2.

29. An agent comprising a targeting moiety which binds specifically to an external domain of Hsp47 expressed on the surface of a cell, in an amount effective to modulate the activity of the cell.

10 30. A pharmaceutical composition, comprising a peptide of claim 24 and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition, comprising an agent of claim 29 and a pharmaceutically acceptable carrier.

15 32. A pharmaceutical composition, comprising an agent comprising a targeting moiety which binds specifically to an external domain of Hsp47 located on the surface of a carcinoma, in an amount effective to generate a cytolytic or cytostatic effect on the carcinoma, and a pharmaceutically acceptable carrier.

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SURFACE LOCALIZED COLLIGIN/HsP47 IN CARCINOMA CELLS

the specification of which

is attached hereto

was filed on 15 MARCH 2000 as United States Application Number or PCT International Application Number PCT/US00/06588 and (if applicable) was amended on _____

I hereby authorize our attorneys to insert the serial number assigned to this application.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 USC §119

APPLICATION NO.	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
60/124,481	US	15 MARCH 1999	YES

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION(S) UNDER 35 U.S.C. §119(e)

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under 35 U.S.C. §120 of any United States application, or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

PRIOR U.S./PCT INTERNATIONAL APPLICATION(S) DESIGNATED FOR BENEFIT UNDER 37 U.S.C. §120

APPLICATION NO.	FILING DATE	STATUS — PATENTED, PENDING, ABANDONED

11 I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith: I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E.J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Richard J. Traverso (30,595); John A. Sopp (33,103); Richard M. Lebovitz (37,067); John H. Thomas (33,460); Catherine M. Joyce (40,668); Nancy J. Axelrod (44,014); James T. Moore (35,619); James E. Ruland (37,432); Jennifer J. Branigan (40,921) and Robert E. McCarthy (46,044)

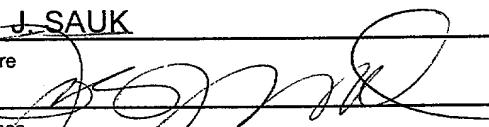
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Additional joint inventors are named on separately numbered sheets attached hereto.